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THE ROLE OF PAK5 IN THE INVASION AND METASTASIS OF UROTHELIAL BLADDER CANCER

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Submitted to fulfil the requirements of the degree of PhD

Abstract

Cytoskeletal regulation in tumour invasion and metastasis involves Ras-related small GTPases and their effectors, such as PAKs. PAK isoforms have been found to be overexpressed in many tumours and are generally associated with poorer prognosis. PAK1 overexpression has been associated with bladder tumour recurrence and progression but the roles for other PAKs are unclear. We found that PAK5 has a distinct expression profile in bladder cancer. We proceeded to characterise the role of PAK5 in the cell biology of bladder cancer.

Bladder cancer cell-lines of invasive and non-invasive origins were screened for the expression of PAK5 using isoform-specific antibody. Confocal microscopy and co-immunoprecipitation were used to characterise the subcellular localisation and interacting partners of PAK5. Functional studies were performed using recombinant PAK5 and siRNA. PAK5 mRNA expression in patient samples was screened by quantitative RT-PCR.

We found that PAK5 protein is differentially expressed in bladder cancer cell lines, with higher expression levels in non-invasive bladder cancer cells. In tissue samples, PAK5 expression was also higher in normal bladder tissues, and reduced in tumours. Endogenous PAK5 co-localises with adherens junction proteins such as E-cadherin and P120-catenin, and their interactions were confirmed by co-immunoprecipitation. Silencing of PAK5 in RT4 cell using siRNA technology affected the cell morphology resulting in smaller and more rounded cells. Silencing of PAK5 also affected the protein level of E-cadherin and the integrity of cell-cell adherens junction.

Our results have indicated that PAK5 expression is associated with non-invasive tumours and cell morphology. We found that in bladder cancer cells, PAK5 is a component of the adherens junction complex. We hypothesise that PAK5 contributes to maintain the adherens junction stability. Further research investigating the mechanism of interaction of PAK5 within the adherens junctions will assess the prognostic and therapeutic utility of PAK5 in bladder cancer.

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My best friend Peter for micromanaging my life during my PhD!

Abbreviations

AID	Auto-inhibitory domain
AJ	Adherens junctions
α-catenin	Alpha-catenin
AR	Androgen receptor
ATP	Adenosine triphosphate
β-catenin	Beta-catenin
Cdc42	Cell division control protein 42 homolog
CRIB	Cdc42/Rac Interactive binding domain
DAPI	4', 6-diamidino-2-phenylindole
E. coli	Escherichia coli
E-cadherin	Epithelial-cadherin
ECM	Extracellular matrix
EGF	Epidermal growth factor
(E)GFP	(Enhanced) green fluorescent protein
EGFR	Epidermal growth factor receptor
EL cells	Mouse L fibroblasts stably expressing E-cadherin
EMT	Epithelial to mesenchymal transition
ERK1	Extracellular receptor kinase 1
ERK2	Extracellular receptor kinase 2
F-actin	Filamentous actin
FBS	Foetal bovine serum
G-actin	Globular actin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF(s)	Guanine nucleotide exchange factor(s)
GTP	Guanosine triphosphate
HGF/SF	Hepatocyte growth factor/Scatter factor
MAPK	Mitogen activated protein kinase
PAK	P21-ras Activated Serine/Threonine Kinase
PBD	p21 binding domain

RAC1	Ras-related C3 botulinum-toxin substrate 1
Mbt	Mushroom bodies tiny
MDCK	Madin-Darby Canine Kidney (cell line)
mRFP1/RFP	Monomeric red fluorescent protein
siRNA	Short interfering RNA
WB	Western blotting/immunoblotting
WCL	Whole cell lysate(s)
WT	Wild-type
X-PAK5	*Xenopus* PAK5 homologue

Chapter 1 - Introduction

1.1 Bladder cancer

1.1.1 Bladder cancer overview

Urothelial bladder cancer (UCB) is a major cause of morbidity and mortality worldwide, causing an estimated 150,000 deaths per year (Jemal, Bray et al. 2011). It is the 7th most common cancer worldwide in men and 17th most common cancer worldwide in women. The incidence of bladder cancer rises with age, peaking between ages of 50 to 70 years. In the European Union, the age standardised incidence rate is 27 per 100 000 men, and 6 per 100 000 in women, and in 2008, UCB was the 8th most common cause of cancer-specific mortality in Europe (Ferlay, Shin et al. 2010). Risk factors for bladder cancer include chemical and environmental exposures to aromatic amines, aniline dyes, nitrites and nitrates, acrolein, coal and arsenic, but the most important environmental factor in current clinical practice is cigarette smoking (Kaufman, Shipley et al. 2009, Rink, Zabor et al. 2013)

Histopathological tumour staging of UCB is by the Tumour-Node-Metastasis (TNM) staging system (approved by the Union International Contre le Cancer (UICC), updated in 2009) in which the primary tumour is assessed on the extent of penetration into the bladder wall, as illustrated in figure 1.1. Approximately 75% of patients with bladder cancer present with non-muscle-invasive bladder cancer (NMIBC) that is either confined to the mucosa (stage Ta, CIS) or to the submucosa (stage T1), while further 25% of patients present with muscle-invasive bladder cancer (MIBC) (Abdollah, Gandaglia et al. 2013). Non-muscle-invasive bladder tumours can be effectively treated in a bladder-conservative approach by transurethral resection and adjuvant intravesical therapy. Patients with non-muscle-invasive bladder cancer have good prognosis, with survival rates of up to 94% (Kaufman, Shipley et al. 2009). However, as many as 50-70% of these superficial tumours can recur, and 20-30% progress to muscle-invasive disease within 5 years of treatment (Rubben, Lutzeyer et al. 1988, Rink, Babjuk et al. 2013). The significant risk of recurrence and progression means that most patients undergo prolonged bladder surveillance (cystoscopy), and in some cases cytological or molecular urinary tests, making NMIBC one of the most expensive human malignancies to manage (Sievert, Amend et al. 2009).

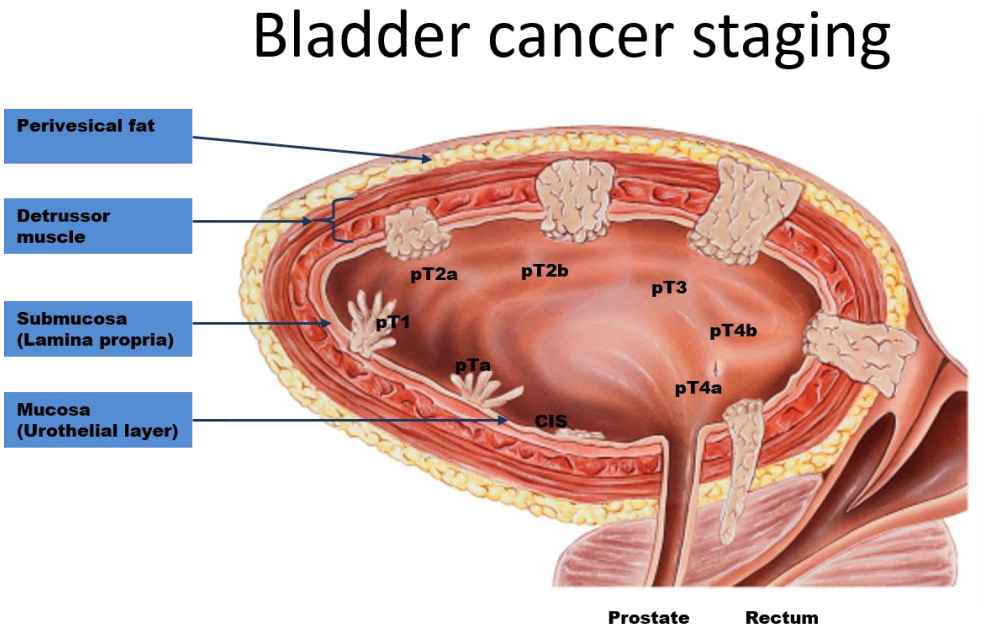

Figure 1-1: Tumour staging for bladder cancer differentiating Non Muscle-Invasive bladder cancer (NMIBC) and Muscle-Invasive Bladder Cancer (MIBC) in men

In contrast, muscle invasive bladder cancer differs significantly in its management, and the associated prognosis compared to NMIBC. The established standard of care for organ-confined bladder cancer invading the muscularis propria/ detrusor muscle is radical cystoprostatectomy for men, and anterior exenteration (removal of the bladder, urethra, uterus and anterior vaginal wall) for women (Kaufman, Shipley et al. 2009, Gakis, Efstathiou et al. 2013). In addition to radical surgery, chemotherapy either in adjuvant or neo-adjuvant setting has the potential to eradicate micrometastasis, and was shown to improve recurrence free and overall survival in patients, especially those with pathologic extravesical and lymph node positive MIBC (Sonpavde and Sternberg 2010). However, despite radical treatment, the prognosis for MIBC is poor, with 5-year overall survival of 36-49%, and 10-year overall survival of 27-32% (Dalbagni, Genega et al. 2001, Stein, Lieskovsky et al. 2001, Grossman, Natale et al. 2003). Invasion of urothelial carcinoma into the surrounding smooth muscle of the bladder wall, and dissemination of metastasis are associated with the worst prognosis leading to significant morbidity and mortality. Patients with locally advanced or metastatic bladder cancer are only expected to have a 5-year survival of 10-15% despite active chemotherapy regimens (von der Maase, Sengelov et al. 2005, Roberts, von der Maase et al. 2006).

In addition to TNM staging, tumour grading is also a very useful prognostic tool in the management of bladder cancer, especially NMIBC, and has been incorporated in the EORTC nomogram to predict the risk of tumour recurrence and progression (Sylvester, van der Meijden et al. 2006). The new classification for grading urothelial carcinomas proposed by the World Health Organisation (WHO) and International Society of Urological Pathology was published in 2004. However, in clinical practice, both the 1973 and 2004 classifications (table 1.1) are simultaneously included in pathological reporting, as attempts to demonstrate better prognostic value of one over the other have yielded controversial results (May, Brookman-Amissah et al. 2010, Pan, Chang et al. 2010). (Otto, Denzinger et al. 2011). It has therefore been recommended that until the WHO 2004 system is validated by more prospective trials and incorporated into prognostic models, both the 1973 and 2004 classifications should be used (Babjuk, Burger et al. 2013).

World Health Organisation (WHO) Grading of Urothelial tumours	
1973 WHO grading	
Urothelial papilloma	
Grade 1	Well differentiated
Grade 2	Moderately differentiated
Grade 3	Poorly differentiated
2004 WHO grading	
Flat lesions	
Hyperplasia (flat lesion without atypia or papillary	
Reactive atypia (flat lesion with atypia)	
Atypia of unknown significance	
Urothelial dysplasia	
Urothelial carcinoma *in situ*	
Papillary lesions	
Urothelial papilloma (benign lesion)	
Papillary urothelial neoplasm of low malignant potential	
Low-grade papillary urothelial carcinoma	
High-grade papillary urothelial carcinoma	

Table 1-1 : Grading of urothelial tumours

One of the disadvantages of the TNM staging and WHO grading systems is the variability of reporting amongst the pathologist, despite the well-defined criteria, and the general conformity in staging and grading is only between 50-60% (Witjes, Moonen et al. 2006, May, Brookman-Amissah et al. 2010). The areas where significant variability have been noted were for the diagnosis of CIS, in the classification of T1 versus Ta tumours, and the tumour grading in both the 1973 and 2004 classifications. This variability can significantly impact the management of patients with NMIBC, where they may be over- or under-treated, both of which encompass high risk of morbidity and mortality. Resources must therefore be allocated to further understand the biology of bladder cancer, in search of molecular markers which can be used as an adjunct to improve the risk stratification of NMIBC, and appropriate selection of treatment or therapeutic target.

Another point of note is that as the outcomes for many types of cancers have improved over the decade but no significant improvement has been noted for bladder cancer (Malmstrom 2011). High recurrence and progression rates still characterise non-muscle-invasive bladder tumours, associated with high mortality as patients with muscle invasive tumours succumb to the disease. Despite complete excision, patients with MIBC have significantly poorer prognosis than patients with NMIBC. This may be explained by the hypothesis that MIBC is a systemic disease, with or without clinical evidence for invasion or metastasis, and extending the boundaries of surgery in these patients may not drastically improve survival (Malmstrom 2011). Further research to understand the cellular and molecular behaviour of urothelial cells leading to MIBC holds the promise of more effective markers and therapeutic targets for bladder cancer.

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1.1.2 Cellular and molecular aberrations associated with urothelial cancer

In order to understand the remarkable diversity of neoplastic diseases, Hanahan and Weinberg (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011) published 2 review articles in 2000 and 2011 on proposed hallmarks of cancer, which together constitute an organising principle for a logical framework in cancer studies. The reviews proposed that as normal cells evolve progressively to neoplastic state, they acquire a succession of hallmark capabilities; these hallmarks of cancer constitute the ability of tumours to sustain proliferative signalling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, and activate invasion and metastasis. In addition to these hallmarks, new developments in cancer research over a decade have added two enabling characteristics (genome instability and mutation, and tumour-promoting inflammation), and two emerging hallmark capabilities (avoiding immune destruction, and deregulating cellular energetics) to the list (Hanahan and Weinberg 2011). These hallmarks complement each other to enable tumour growth, invasion and metastasis. By directly or indirectly applying the logical framework proposed by Hanahan and Weinberg, several studies have attempted to characterise the molecular pathways and biomarkers associated with bladder cancer. The biomarkers that have been extensively studied are listed in table 1.2.

Pathway	Biomarker
Cell cycle	p53, pRb, Ki-67, p21 (CIP1/WAF1), p27, Cyclins
Apoptosis	Fas (CD95), Caspace-3, Bcl-2, Survivin
Angiogenesis	Thrombospondin-1, VEGF, bFGF
Signalling proteins	EGFR, HER2, FGFR3
Hormone receptors	Androgen receptor, Oestrogen receptor

Table 1-2: Extensively studied immunohistochemical biomarkers in bladder cancer, adapted from the following reviews (Bryan, Zeegers et al. 2010, Matsushita, Cha et al. 2011, Kamat, Hegarty et al. 2013)

The hallmarks of cancer, as proposed by these reviews have been challenged as a valid strategy in the understanding of bladder cancer (Malmstrom 2011). In the development of bladder cancer, clinical and pathological studies indicate that there are two distinct subtypes of bladder cancer; NMIUC/low grade and MIUC/high grade tumours. Urothelial carcinoma of the bladder is unique amongst epithelial cancers in its divergent pathways of tumorigenesis (figure 1.2), based on the genetic aberrations, biological behaviour and prognosis (Koss 1998, Wu 2005, Knowles 2006). The only common aberration shared by both low grade and high grade urothelial carcinomas is the deletion of both arms of chromosome 9, which occurs at the earliest stage of tumorigenesis, and these chromosomal aberrations do not distinguish between the two tumour developmental pathways (Stoehr, Zietz et al. 2005).

Consequently, some tumours progress from urothelial hyperplasia to low-grade non-invasive superficial papillary tumours. Low grade papillary tumours rarely become muscle invasive, and they frequently harbour gene mutations that constitutively activate the receptor tyrosine kinase-Ras pathway (Dinney, McConkey et al. 2004), as well as mutations of fibroblast growth factor receptor 3 (FGFR3) oncogene (Cappellen, De Oliveira et al. 1999).

By contrast, more aggressive variants arise either from flat high grade carcinoma *in situ* and progress to invasive tumours, or they arise *de novo* as invasive tumours. Most high-grade invasive tumours progress to life threatening metastasis and have defective p53 and/or Retinoblastoma protein (pRB) tumour-suppressor pathways. (Cordon-Cardo, Dalbagni et al. 1994, Spruck, Ohneseit et al. 1994, Orntoft and Wolf 1998, Billerey, Chopin et al. 2001, Lu, Wikman et al. 2002, Wu 2005) .

Tumour invasion and progression in bladder cancer appear to be parts of multifactorial process, promoted by intracellular and micro-environmental changes that include the upregulation of N-Cadherin and the corresponding downregulation of E-cadherin (Garcia del Muro, Torregrosa et al. 2000, Nakopoulou, Zervas et al. 2000, Popov, Gil-Diez de Medina et al. 2000, Bryan, Atherfold et al. 2008), the overexpression of matrix metalloproteinases 2 and 9 (Kanayama 2001, Slaton, Karashima et al. 2001), and imbalance between angiogenic and anti-angiogenic factors (Grossfeld, Ginsberg et al. 1997, Komhoff, Guan et al. 2000).

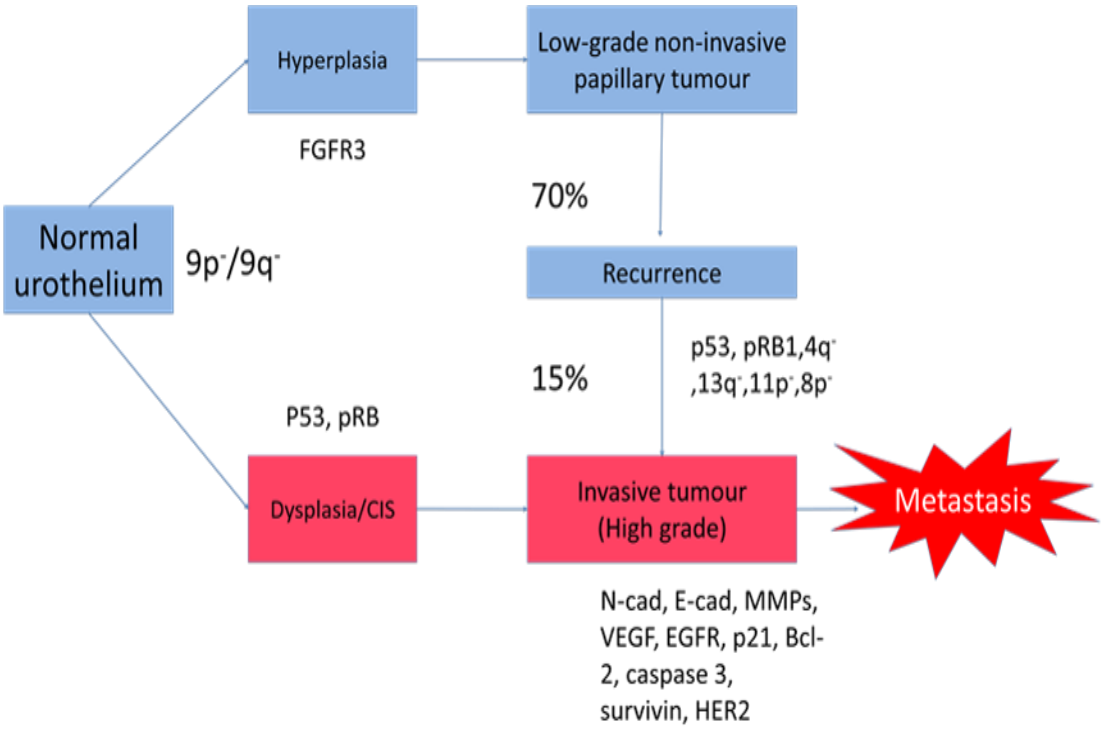


Figure 1-2 : Divergent pathway of urothelial tumorigenesis. Adapted from reviews (Wu 2005, Knowles 2006, Bolenz and Lotan 2008)

Despite the insights that have been obtained into the mechanisms involved in the cell biology of bladder cancer, the disease specific mortality has changed very little over the last 20 years in Western Europe and the USA (Whelan 2008). As most of the cancer mortality is associated with MIBC, further research in the regulation of urothelial cancer invasion and metastasis is warranted. Progression to, or presentation with muscle invasive disease represents the critical step in the disease course of bladder cancer. Thus, detailed biological and molecular insights into the pathogenesis of high grade, muscle invasive bladder cancer are urgently needed so that the disease can be more adequately treated at presentation, progression from non-muscle invasive stages can be abrogated, and the risk of recurrence after radical treatment can be minimised. A controversial, yet extensively studied process that may have a crucial role in the divergent pathways in bladder cancer is epithelial to mesenchymal transition, further discussed in the next section.

1.1.3 An overview of epithelial to mesenchymal transition as a precursor to bladder cancer invasion and metastasis

Epithelial to mesenchymal transition (EMT) is a biologic process during embryologic development in many animal species by which polarised epithelial cells lose their characteristic cell polarity, disassemble cell-cell junctions and become more migratory as they assume mesenchymal cellular phenotype (Yang and Weinberg 2008, Kalluri and Weinberg 2009). EMT has been observed in physiological, as well as pathological processes in cancer biology associated with tumour invasion, migration and metastasis (Thiery 2002, Tam and Weinberg 2013). In the context of cancer biology, Cadherins represent an important aspect of EMT, where the normal expression of epithelial Cadherin (E-cadherin), is suppressed, and switched to neural Cadherin (N-Cadherin) or placental Cadherin (P-Cadherin), a process known as Cadherin switching (Cavallaro, Schaffhauser et al. 2002).

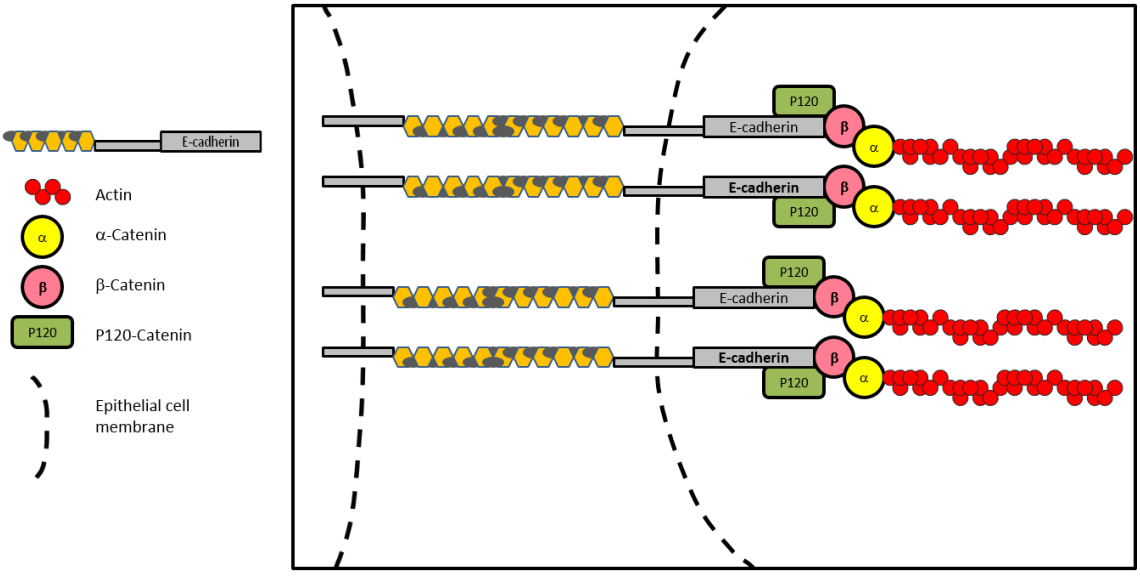


Figure 1-3 : Schematic diagram of cadherin-catenin protein complex at cell-cell adherens junction

The presence of adherens junctions (AJs) is a defining feature of all epithelial tissue (Fristrom 1988, Baum and Georgiou 2011). The prototypical protein that forms the transmembrane core of adherens junctions is E-cadherin. Formation of cell-cell adherens junctions requires interactions of the cytoplasmic tail of the E-Cadherin molecule in complex with p120-, α- and β-Catenins with

the actin cytoskeleton (figure 1.4). Whilst the extracellular domain of E-Cadherin is responsible for homotypic, calcium-dependent interactions with E-Cadherins on the surface of adjacent cells, the cytoplasmic domain of E-Cadherin interacts with p120-Catenin and β–catenin through its juxtamembrane and catenin-binding domains respectively. β–catenins provides linkage between adherens junctions and the actin cytoskeleton through interactions involving α–catenin (Vasioukhin and Fuchs 2001, Nelson 2008).

Decreased E-cadherin immune-reactivity was first described in bladder cancer in 1993 (Bringuier, Umbas et al. 1993). A number of studies then followed, which demonstrated cadherin switching in the setting of bladder cancer, associated with late stage, high grade disease (Rieger-Christ, Cain et al. 2001, Clairotte, Lascombe et al. 2006, Lascombe, Clairotte et al. 2006, Bryan, Atherfold et al. 2008, Mandeville, Silva Neto et al. 2008). Rieger-Christ et al observed that N-Cadherin expression was not detected in normal urothelium, but was localised to the membrane in focal areas of tumour mass in the majority (61%) of invasive bladder cancer. In addition to N-cadherin, membranous P-Cadherin was expressed throughout the tumours in high grade disease, where E-cadherin expression was decreased or absent.

A detailed study on cadherin-switching using pT1 and T2-T3 bladder tumour samples (Lascombe, Clairotte et al. 2006) demonstrated that N-Cadherin expressing bladder cancer progressed more rapidly, and the majority of T2-T3 tumours demonstrated no expression of E-cadherin. Furthermore, Bryan et al (Bryan, Atherfold et al. 2008) investigated P-Cadherin in 153 human bladder cancer specimen, and observed that in high grade, muscle invasive tumours, E-Cadherin expression was decreased, P-Cadherin expression was increased, and this cadherin switching was associated with worse bladder cancer specific survival. Functional in vitro models by Bryan et al demonstrated profound changes when E-Cadherin and P-Cadherin expression were manipulated, where P-Cadherin alone was unable to mediate normal cell-cell adhesion, and altering the balance of E-Cadherin and P-Cadherin, with decreases E-Cadherin expression enhanced anchorage independent cell growth, one of the prerequisites for invasive, metastatic phenotype.

P120-catenin is a cytoplasmic molecule closely associated with E-cadherin at cell-cell adherens junction, and p120-Catenin and E-Cadherin are expressed in membranous pattern in normal bladder epithelium. Loss of normal surface P120-catenin has be reported in bladder tumours, with

significant correlation to tumour grade, stage and poor survival (Syrigos, Karayiannakis et al. 1998, Silva Neto, Smith et al. 2008). These studies have also noted that in comparing the pattern of expression of p120-Catenin and E-cadherin from the same tumour samples, there was a high frequency of simultaneous occurrence of E-Cadherin loss with abnormal expression or relocalisation of p120, and the prognosis was worse for cancer specific survival when p120 and E-Cadherin loss of expression were grouped together.

The regulatory mechanisms of cadherin switching in bladder cancer remains unclear, but they most likely involve transcriptional and post transcriptional events, and influenced by cytokines or growth factors associated with EMT (Wheelock, Shintani et al. 2008). It can therefore be postulated that EMT does not just represent a change in cell-cell adhesion, but a rather fundamental reorganisation of cell biology, involving changes to the cytoskeleton, membrane protrusions and extracellular microenvironment (Yilmaz and Christofori 2009). P21-activated Serine/Threonine Kinases (PAKs), a family of Rho-GTPases effectors which regulate the actin cytoskeleton could potentially provide further insights into the regulation of EMT in bladder cancer to enable well-directed diagnosis and targeted management of high risk bladder cancer in the age of personalised cancer care. An overview of Rho-GTPases, PAKs, actin cytoskeleton and their integration into the process of EMT in cancer cell invasion and metastasis is discussed in the next sections

1.2 P21-Activated Serine/Threonine Kinases (PAKS)

The invasion of cancer cells into the surrounding tissue is a prerequisite and initial step in metastasis. Invasive cell migration requires the formation of various cellular structures, which require actin assembly and cytoskeletal organisation (Sit and Manser 2011). Actin forms the cellular scaffold structure that provides cells with their shape, tension support, intracellular vesicular transport, cell attachment, adhesion properties and the ability to move. There are 4 distinct ways of extending the membrane at the leading edge; lamellopodia, filopodia, invadopodia and membrane blebs (Ridley 2011).Each protrusion requires the coordination of wide spectrum of signalling molecules and regulators of cytoskeletal dynamics.

1.2.1 Rho family GTPases regulation of actin cytoskeleton and cell: cell adhesion

The Rho-subfamily of GTPases regulates the local assembly or disassembly of filamentous (F)-actin in the cytoskeleton (Hall 1998). The best-characterised Rho-GTPases are RhoA, Rac and Cdc42. Most Rho-GTPases act as molecular switches, cycling between GTP-bound active form and GDP-bound inactive forms. The activity is increased by guanine nucleotide exchange factors (GEFs) and down regulated by GTPase-activating proteins (GAPs). Guanine nucleotide dissociation inhibitors (GDIs) regulate the activity of Rho-GTPases by binding to the C-terminal prenyl group, preventing Rho-GTPase membrane association, and thus inhibits access to their effectors (Vega and Ridley 2008)

Rho family of GTPases; Rho, Rac and Cdc42 are well-known regulators of actin dynamics with important roles in intercellular adhesion involving cadherin-catenin complex in epithelial cells (McCormack, Welsh et al. 2013). Seminal papers on the formation of cell-cell adherens junction in epithelial models had emphasised that the changes to the cell membrane protrusions as a regulated process, demonstrating the roles for Rho-GTPases as the master regulators of actin cytoskeleton necessary for stable cell-cell adhesion (Vasioukhin, Bauer et al. 2000, Vasioukhin and Fuchs 2001, Vaezi, Bauer et al. 2002) (Braga, Machesky et al. 1997, Adams, Chen et al. 1998, Vasioukhin, Bauer et al. 2000). The spatio-temporal control of small GTPase activation drives specific intracellular processes to enable hierarchical assembly, morphology and maturation of cell-cell contacts. Dysregulation of Rho GTPases has been demonstrated to induce

cell-cell dissociation or cell migration, implicated in the process of cancer invasion and metastasis (Kuroda, Fukata et al. 1998, Reymond, Im et al. 2012).

The current knowledge on the regulation of cell-cell junctions is derived from well-characterised RhoA, Rac1 and Cdc42. (Niessen, Leckband et al. 2011). Rac1 is required for the recruitment of F-actin to clustered cadherin receptors (Braga, Machesky et al. 1997). RhoA is necessary for the formation and function of adhesive complexes, and its interactions with p120-Catenin in adherens junctions have been well characterised (Anastasiadis, Moon et al. 2000, Reynolds and Roczniak-Ferguson 2004). However, conflicting results have demonstrated both activation and inactivation of RhoA by cadherin-mediated cell-cell adhesions (Calautti, Grossi et al. 2002, Noren, Arthur et al. 2003, Yamada and Nelson 2007). The functions of Cdc42 in epithelial morphogenesis and cell-cell adhesion has been well characterised, with distinct roles in the formation of filopodia which serves as the sites of actin polymerisation in the initiation of adherens junction formation(Vasioukhin, Bauer et al. 2000), stabilisation and localisation of cadherin-catenin complexes to adherens junctions, in part, by regulation of endocytic trafficking of adhesion and polarity proteins (Harris and Tepass 2008). Most importantly, although Rho, Rac and Cdc42 are required in adherens junction assembly, the hyperactivation of the same GTPases may also destabilise the epithelial junctional morphology, a mechanism implicated in cancer progression and metastasis (Braga, Betson et al. 2000, Bray, Gillette et al. 2013, Ridley 2013).

Activated Rho-GTPases bind to a variety of effectors that regulate these cytoskeletal dynamics, and P21-activated kinases (PAKs) are one family of such effectors (Manser, Leung et al. 1994). Although PAK1 has been implicated in the recurrence of urothelial cancer of the bladder (Ito, Nishiyama et al. 2007) and progression of upper tract urothelial cancer (Kamai, Shirataki et al. 2010), the role of other PAKs have not been well characterised in urothelial oncogenesis.

1.2.2 PAK domain structure and regulation

PAKs have been well characterised as signal transducers, central to many vital processes in physiology and disease, including cell morphology, motility, survival, gene transcription and hormone signalling. PAK (PAK1) was first identified in a screen for RAC and Cdc42 effectors (Manser, Leung et al. 1994). In the decade following the cloning and characterisation of PAK1, additional members of the human PAK-family began to emerge with the identification of PAK-isoforms 2-6. PAK family of kinases are architecturally similar and their structure can be divided into three main domains: an N-terminal PBD (p21-GTPase binding domain), a central region and a highly conserved C-terminal serine/threonine kinase domain (Kumar, Gururaj et al. 2006, Wells and Jones 2010). PAK-isoforms are subdivided into two groups based on structural and functional features (Jaffer and Chernoff 2002, Molli, Li et al. 2009); group-1 consists of PAK1, PAK2 and PAK3, while group-2 consists of PAK4, PAK5 and PAK6 (figure 1.2).

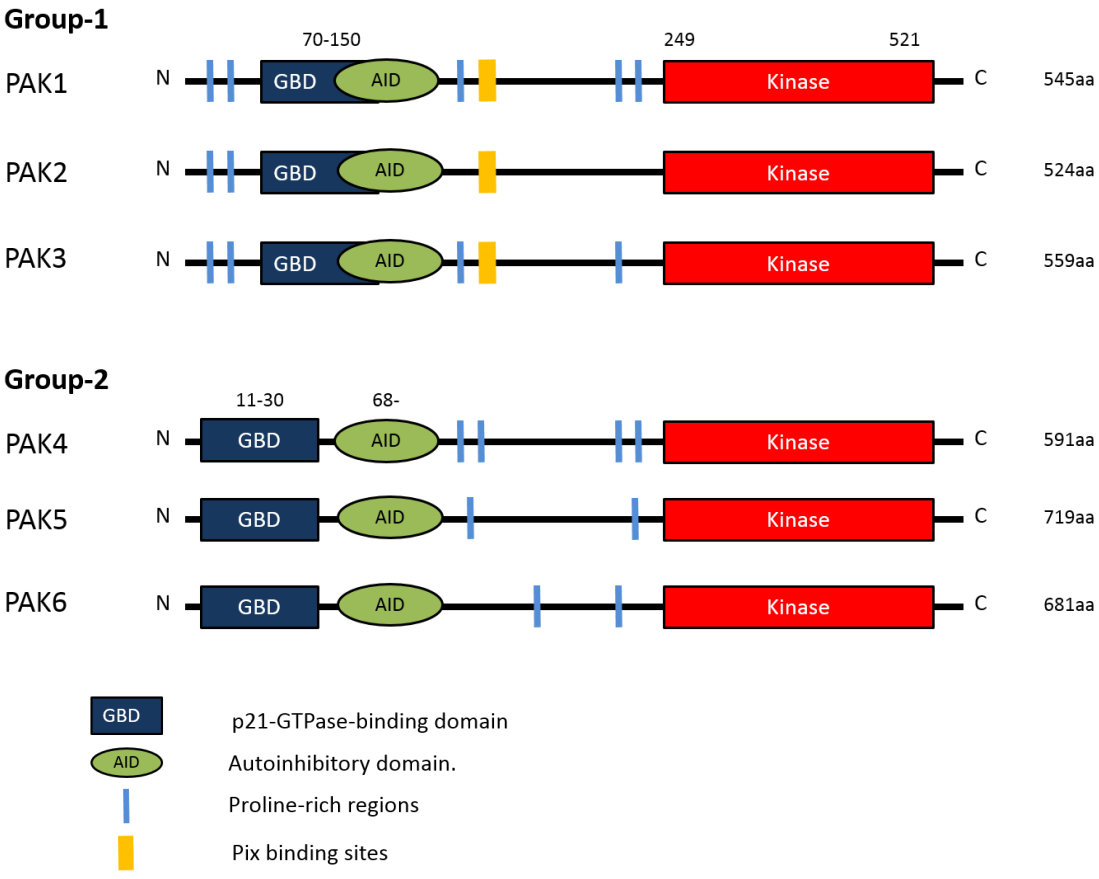


Figure 1-4 : Structural domains of groups 1 and 2 PAKs showing N-terminal regulatory domain and C-terminal kinase domain

The regulatory domain of group-1 PAKs contain a conserved p21-GTPase binding domain (GBD) and auto-inhibitory domain (AID). Group 1 PAKs form homo-dimers in cells, adopting a trans-inhibited conformation where the N-terminal AID of one molecule binds and inhibits the catalytic domain of the other (Lei, Lu et al. 2000). Binding of activated Rho GTPases to the PBD disrupts dimerization and activates group 1 PAKs. This releases the AID-mediated inhibition and allows autophosphorylation of the activation loop, which as a result, prevents refolding and consequent inhibition, and when the phosphorylated kinase domain binds to a substrate, it adopts a monomeric conformation, which is the active catalytic state (Buchwald, Hostinova et al. 2001, Pirruccello, Sondermann et al. 2006).

The PBD/CRIB domain is conserved in group-2 PAKs, but the roles of Rho-GTPase interaction at this site remains under intensive scrutiny. It had been initially postulated that group-2 PAKs lack AID, as PAK4 and PAK6 showed no increased kinase activity on GTPase binding as typified by group 1 PAKs (Yang, Li et al. 2001, Pandey, Dan et al. 2002). Contrary to initial postulations, evidence for distinct autoinhibitory domain in group-2 PAKs have become more consolidated. Although the marked kinase activity observed in group-1 PAKs upon binding to Rho GTPases has not been demonstrated in group-2 PAKs, co-expression of Rho GTPases, such as Cdc42, with group-2 PAKs resulted in dramatic consequences, often observed to be associated with changes in group-2 PAKs subcellular localisation (Abo, Qu et al. 1998{Wu, 2006 #5453, Wu and Frost 2006).

Group 2 PAKs have been demonstrated to possess higher basal kinase activity than group 1 PAKs (Wells, Abo et al 2002). It had also been demonstrated that the kinase activity of group 2 PAKs are significantly higher in the truncated versions which only contain the kinase domains (lacking the regulatory domain), compared to the full length proteins (Abo, Qu et al. 1998, Yang, Li et al. 2001). In relation to Rho GTPase regulation/interaction, although PAK4 and PAK6 are still able to bind to CDC42, the binding does not enhance their kinase activity (Abo, Qu et al. 1998, Dan, Nath et al. 2002, Pandey, Dan et al. 2002, Wells, Abo et al. 2002). In addition, PAK5 has also been found to bind to Cdc42, and a study has identified an auto-inhibitory fragment of PAK5 that is absent from PAK4 and PAK6 (Ching, Leong et al. 2003). Taken together, these results suggest that group 2 PAKs are regulated intra-molecularly, but through different mechanisms to group 1 PAKs.

1.2.3 PAKs in cell motility and invasion

PAKs occupy a central position in oncogenic signalling, and drive several hallmarks of cancer, which include activating cell invasion and metastasis (reviews: (Molli, Li et al. 2009, Radu, Semenova et al. 2014). The initial stages of cancer invasion involve extensive remodelling of the cytoskeleton, disruption of cell adhesions and release of proteases that break down the extracellular matrix. PAKs have an important role in the regulation of these events, usually by mediating responses to Rho family GTPases such as Rac or Cdc42, by phosphorylation of downstream PAK substrates that control cytoskeletal dynamics.

Group 1 PAKs have been implicated in cell migration through their ability to phosphorylate multiple cytoskeletal regulators (Arias-Romero and Chernoff 2008). In epithelial cells, PAKs may contribute to both lamellopodial extension and disruption of cell-cell junctions. For example, PAK1 is activated by HGF in MDCK cells and expression of the N-terminal regulatory domain of PAK2 as a dominant negative blocks HGF-induced peripheral actin remodelling and lamellopodium extension (Royal, Lamarche-Vane et al. 2000). LIM domain Kinase 1 (LIMK1) is one of the most established PAK1 substrates which directly regulate the actin cytoskeleton. PAK1 phosphorylates LIMK1 at Threonine residue 508, which stimulates its activity (Edwards, Sanders et al. 1999).

Rac1/PAK1/LIMK1 signalling pathway controls cofilin activity within the lamellapodium by phosphorylation at Ser3 on its N-terminal, and the enhancement of cofilin activity accelerates F-actin turnover and retrograde flow, resulting in widening of the lamellapodium (Delorme, Machacek et al. 2007). This pathway of Rac1 induced actin reorganisation may be specific to particular PAK isoforms. In breast carcinoma, PAK1 but not PAK2 mediates the formation of heregulin-stimulated lamellapodial protrusions, the maturation of focal adhesions, cofilin phosphorylation and the loss of RhoA activity (Coniglio, Zavarella et al. 2008). A similar dichotomy regarding PAK isoforms and cytoskeletal activity has also been demonstrated in mast cells, in which PAK1 and PAK2 have opposing roles in actin organisation and degranulation(Allen, Jaffer et al. 2009, Kosoff, Chow et al. 2013).

In prostate cancer cells, HGF has also been demonstrated to stimulate phosphorylation of PAK1 and PAK2, and knockdown of PAK1 inhibits HGF-stimulated migration in DU145 cells, whereas knockdown of PAK2 increases lamellapodium extension but not migration speed (Bright, Garner

et al. 2009). The study by Bright *et al* also reported that PAK2 knockdown increased phosphorylation of PAK1, which indicates that PAK2 provides a negative feedback on PAK1.

Group 2 PAKs have also been characterised in the regulation of actin cytoskeleton to mediate changes in cell motility, adhesion and morphology, of which PAK4 is the well-characterised isoform. As the founding member of group 2 PAKs, PAK4 was demonstrated to induce filopodia formation downstream of Cdc42 (Abo, Qu et al. 1998). Overexpression of PAK4 has also been shown to result in the dissolution of stress fibres and cell rounding, which is accompanied by loss of focal adhesions (Wells, Abo et al. 2002, Barac, Basile et al. 2004). Similar to PAK1, PAK4 also has the capacity to regulate actin depolymerisation through the phosphorylation of LIMK1 on Threonine residue 508, which in turn phosphorylates cofilin (Dan, Kelly et al. 2001, Ahmed, Shea et al. 2008). PAK4 forms a multiprotein complex with LIMK1, Slingshot phosphatase (SSH-1L) and the scaffolding protein 14-3-3zeta, and within this signalling pathway, PAK4 activates LIMK1 and inactivates SSH-1L, resulting in actin filament turnover through an increase in cofilin activity (Soosairajah, Maiti et al. 2005).

PAK4 has also been shown to regulate cell focal adhesions, where activated PAK4 expression causes loss of focal adhesion, and cells with reduced PAK4 expression have increased focal adhesions (Wells, Abo et al. 2002, Wells, Whale et al. 2010). This is consistent with the finding that cells from PAK4 knockout mice also showed elevated levels of focal adhesions, suggesting dysregulation of adhesion molecules in the absence of PAK4 (Qu, Li et al. 2003). PAK4 may regulate focal adhesions either through PAK4-mediated phosphorylation of Paxillin on Serine 272 (Wells, Whale et al. 2010), or by the phosphorylation of the cytoplasmic tail of integrin $\alpha v\beta 5$ (Li, Lock et al. 2010). Activated PAK4 induces accelerated integrin $\alpha v\beta 5$ turnover within the adhesion, which in turn inhibited integrin $\alpha v\beta 5$ clustering, reduced integrin to F-actin connectivity and perturbed focal adhesion complex maturation, which ultimately lead to reduced cell adhesion strength and increased cell motility (Li, Lock et al. 2010).

A number of studies had supported the role for PAK4 in cancer invasion and metastasis. Expression of constitutively active PAK4 in pancreatic ductal cells resulted in an increase migratory capacity and invasion, whilst PAK4 siRNA knockdown reduced invasion in an in *vitro* assay (Kimmelman, Hezel et al. 2008). PAK4 was also shown to have a role in migration and adhesion of prostate cancer cells (Wells, Abo et al. 2002, Ahmed, Shea et al. 2008, Wells, Whale

et al. 2010). Studies by Wells et al demonstrated the regulation of PAK4 downstream of HGF, which in turn mediated loss of cell-cell adhesion and cell migration through activation of LIMK1. The level of auto-phosphorylated PAK4 is elevated in prostate cancer cells in response to HGF, and when PAK4 levels are reduced using siRNA, the cells become deficient in their invasive and migratory abilities in response to HGF. Taken together, these data point to an important link between PAK4 and EMT in a number types of cell from different epithelial tissues, closely related to the role of PAK4 in the regulation of actin cytoskeleton and cell adhesion.

The reorganisation of the extracellular matrix is also required for tumour cell invasion to provide space for cell movement (Gupta and Massague 2006), and the destruction of the extracellular matrix is partly controlled by the release of matrix metalloproteinases (MMP). It is likely that PAKs mediate certain aspects of extracellular matrix reorganisation downstream of Cdc42, as matrix remodelling could not be restored to $Cdc42^{-/-}$ mouse embryonic fibroblasts (MEFs) by reintroducing mutants of Cdc42 that lacked PAK binding capability(Sipes, Feng et al. 2011). Increased expression of MMPs has been suggested to result from PAK-mediated activation of Jun N-terminal kinase (Zhou, Yan et al. 2009), and PAK1, PAK2, PAK4 and PAK5 have been shown to regulate MMP expression in various cancer cell types, where group 1 PAKs was shown to regulate MMP9 (Goc, Abdalla et al. 2012, Rider, Oladimeji et al. 2013), whereas group 2 PAKs interact with MMP2 (Kesanakurti, Chetty et al. 2012, Wang, Cheng et al. 2013).

Cancer invasion involve extensive remodelling of the cytoskeleton for cell motility and migration, and release of proteases that break down the extracellular matrix. In epithelial cancers such as urothelial carcinoma of the bladder, the disruption of cell-cell adhesions may play a significant role in differentiating low risk against high risk bladder tumours with great impact on prognosis (reviewed in section 1.1.3). Therefore, a detailed review on the role of PAKs in the regulation of epithelial cell-cell adhesions is provided in the section 1.2.4.

1.2.4 **PAKs and cell-cell adhesions**

PAK family of proteins, downstream effectors of Rho GTPases in the signalling pathways in the regulation of actin cytoskeleton, have been demonstrated to be directly involved with formation, or dissociation of epithelial cell-cell junctions. The most extensively studies group-1 PAKs, PAK1, has been shown to modulate the cadherin-catenin complex at the cell-cell adherens junction. PAK1 kinase mutants have also been shown to induce loss of cell-cell junctions in MDCK cells, and active Rac1 signals via PAK1 to induce disassembly of E-cadherin based adhesions in keratinocytes (Zegers, Forget et al. 2003, Lozano, Frasa et al. 2008). PAK1 has been shown to promote transcription-repression activity of Snail for E-Cadherin (Yang, Rayala et al. 2005). In the study by yang et al, PAK1 phosphorylation of Snail on Ser246 was demonstrated to be one of the mechanisms for downregulation of E-cadherin, and EMT associated with invasion and metastasis in breast cancer.

The evidence for the role of group-2 PAKs in cell adhesion is emerging, albeit poorly understood. One of the studies has demonstrated that Cdc42 regulates the apical junction formation in human bronchial epithelial cells through PAK4 (Wallace, Durgan et al. 2010). The conclusion of this study may appear contradictory, as PAK4 was found to be necessary for the formation of cell-cell apical junctions in bronchoepithelial cells, but the expression of constitutively active PAK4 caused cell-cell dissociation. It could therefore be hypothesised that tight control in the kinase activity or spatial regulation of PAK4 by Cdc42 is necessary in the maintenance and formation of cell adhesions.

PAK6, which was initially identified to be an androgen receptor protein, has recently be reported to be involved in cell-cell dissociation in response to HGF independent of androgen receptor (AR) signalling (Fram, King et al. 2014). In the study, Fram et al demonstrated that PAK6 was required for AR-deficient DU145 prostate cancer cells and HT29 colon cancer cells to dissociate and scatter following HGF stimulation, and PAK6 overexpression resulted in destabilisation of cell-cell adhesion, independent of growth factor (HGF) stimulation. Mechanistically, a complex of PAK6/E-cadherin/IQGAP1 was identified as the cells dissociate in response to HGF, and the level of PAK6 auto-phosphorylation was elevated in the presence of IQGAP1. The in *vitro* kinase assay performed in the study did not show phosphorylation of IQGAP1 by PAK6, but identified β-Catenin

to be a novel substrate for PAK6, and may be involved in cell-cell dissociation by phosphorylation of serine-675 downstream of PAK6.

The interactions between PAK6 with E-Cadherin and β-Catenin described by Fram *et al* poses the question whether PAK6 may also interact with other members of adherens junction Cadherin-Catenin complex such as p120-catenin. This question had already been addressed in a study on Group 2 PAKs interactions with p210-Catenin, where in contrast to PAK4 (constitutively active) and PAK5 (wild-type and constitutively active), neither wild-type nor constitutively active PAK6 showed distinct interaction with p120-Catenin (Wong, Reynolds et al. 2010). The findings of this study are further discussed in the following sections.

1.3 PAK5, a novel member of the PAK-family

PAK5 is a novel, and the least understood member of the PAK-family of proteins. The gene encoding for PAK5 is located on chromosome 20 at 20p12. It is structurally most related the members of group-2 PAK subfamily (Dan, Nath et al. 2002, Pandey, Dan et al. 2002). Unlike group-1 PAKs (PAK1, PAK2 and PAK3) which are quite similar to each other throughout their sequences, PAK4, PAK5 and PAK6 only share up to 60% of their sequence homology (Jaffer and Chernoff 2002).

High expression of PAK5 mRNA was detected in the brain on Northern blot and *in situ* hybridisation, with moderate expression in a number of epithelial tissues (Pandey, Dan et al. 2002). It had also been noted that in mouse brain development, PAK5 was preferentially expressed in mature neural system than developing ones (Dan, Nath et al. 2002, Pandey, Dan et al. 2002). In addition to neural tissues, PAK5 has also been found to be moderately expressed a selection of normal and malignant human epithelial tissues (Dan, Nath et al. 2002, Pandey, Dan et al. 2002, Gong, An et al. 2009). Although the expression of PAK5 in bladder tissues has not been well characterised, PAK5 expression has been described to be expressed at moderate levels in both normal human bladder and human urothelial tumours of varying grades and stages (http://www.proteinatlas.org/ENSG00000101349/tissue/urinary+bladder, http://www.proteinatlas.org/ENSG00000101349/cancer/tissue/urothelial+cancer).

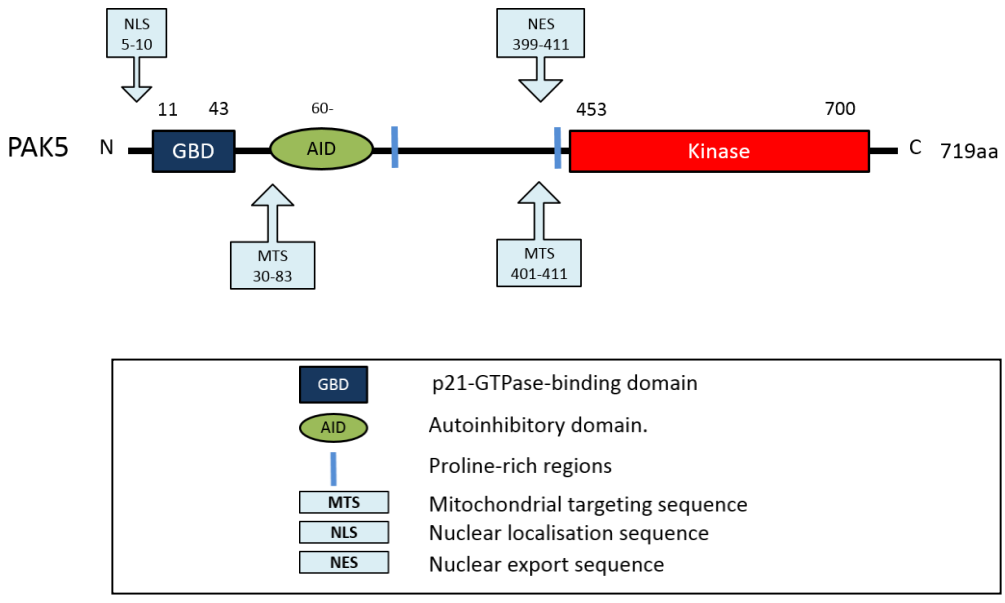


Figure 1-5 : The domain structure of Human PAK5

1.3.1 PAK5 regulation

Similar to other members of the PAK family, PAK5 has a conserved PBD: AID, and has been shown to interact with a number of Rho GTPases (table 1.3). PAK5 preferentially binds to Cdc42 in the presence of GTP, and the AID motif is essential for this interaction (Pandey, Dan et al. 2002). The AID domain within PAK5 is critical for proper targeting within the cell. In addition to Cdc42, PAK5 was also observed to interact with RhoD and RhoH. The interaction with RhoD targets PAK5 to subcellular locations different from those stimulated by Cdc42 (Cotteret, Jaffer et al. 2003, Wu and Frost 2006). Unlike group 1 PAKs, PAK5 does not bind with wild-type Rac1, and only very weakly to constitutively active Rac1 V^{12} (Dan, Nath et al. 2002).

Rho GTPase	Reference	Affinity	Notes
Cdc42	(Wu and Frost 2006) (Dan, Nath et al. 2002) (Pandey, Dan et al. 2002)	+++	Formation of filopodia, Targets PAK5 to the cell membrane
Rac	(Dan, Nath et al. 2002) {Pandey, 2002 #5459	(+)	Very weak interaction noted
RhoD	(Wu and Frost 2006)	++	Targets PAK5 to the mitochondria
RhoH	(Wu and Frost 2006)	+	Binding to constitutively active RhoH was observed but not functionally investigated

Table 1-3: Rho GTPases interaction and regulation of PAK5

A number of N-terminal regions have been identified that regulate the localisation of PAK5: a mitochondrial targeting sequence, a nuclear export sequence, and a nuclear localisation sequence. Deletion of mitochondrial and nuclear transport sequences causes PAK5 to be retained in the nucleus and suppresses PAK5 cellular activity (Cotteret and Chernoff 2006). Localisation of PAK5 to mitochondria has been well characterised and associated with the regulation of apoptosis (Cotteret, Jaffer et al. 2003, Cotteret and Chernoff 2006, Wu and Frost 2006).

1.3.2 **PAK5 substrates**

Protein	Site	Pathway	Reference
Raf-1	Serine 338	ERK/MAPK	(Wu, Carr et al. 2008)
BAD	Serine 112	Apoptosis	(Cotteret, Jaffer et al. 2003)
MARK/Par-1	Kinase-independent inhibition	Cytoskeletal regulation	(Matenia, Griesshaber et al. 2005)
p120-Catenin	Serine 288	Cell adhesion	(Wong, Reynolds et al. 2010)
Pacsin1	Serine 1291	Vesicle trafficking	(Strochlic, Concilio et al. 2012)
Synaptojanin1	Serine 343	Vesicle trafficking	(Strochlic, Concilio et al. 2012)

Table 1-4 Validated PAK5 substrates or interacting partners

Both group 1 and group 2 PAKs have been implicated in apoptosis. PAK1, PAK4 and PAK5 possessed anti-apoptotic properties whereas PAK2 have been shown to be pro-apoptosis. Similar to PAK1 and PAK4, PAK5 inhibits apoptosis by phosphorylates BAD on Serine 112, and this function requires PAK5 localisation to the mitochondria (Cotteret and Chernoff 2006).

Another substrate of PAK5 which requires localisation to mitochondria is Raf-1. Raf-1 is an important effector of Ras-mediated signalling, and is a critical regulator of the ERK/MAPK pathway. PAK5 phosphorylates Raf-1 on the obligate Serine 338 most efficiently compared to other group 2 PAKs (PAK4 and PAK6), and the interaction of PAK5 with Raf-1 targets a subpopulation of Raf-1 to the mitochondria (Wu, Carr et al. 2008). In the same study, a very interesting observation was made, where despite the efficient phosphorylation of Raf-1 by PAK5, mitochondrial targeting of Raf-1 by PAK5 interaction was independent of Serine 338

phosphorylation. These findings have given rise towards the speculation of a kinase-independent, or scaffolding role for PAK5 in the regulation of its interacting partners.

The kinase-independent interaction of PAK5 has since been further characterised in its inhibition of MARK/Par-1 in neuronal cells in *vitro* and in *vivo* (Matenia, Griesshaber et al. 2005). MARK/Par-1 is a kinase involved in the development of embryonic polarity. PAK5 has been shown to suppress the kinase activity of MARK2 towards its target; tau protein, and the inhibition requires the binding between the kinase domains of both PAK5 and MARK2. This inhibition does not require phosphorylation, and this interaction appears to depend on the conformational states of the proteins. The phosphorylation- independent interaction of PAK5 with MARK2 in neuronal cells is functionally significant in the regulation of microtubules and actin cytoskeleton; resulting in stable microtubules, and dynamic actin (loss of stress fibres, dissolution of focal adhesion and formation of filopodia). In contrast to the previously described constitutive subcellular localisation of PAK5 to mitochondria (Cotteret, Jaffer et al. 2003, Wu, Carr et al. 2008), the interaction between PAK5 and MARK2 was localised to the cell membrane, neurites and distinct vesicular structures apart from the mitochondria. This observation hints that the subcellular localisation and spatial regulation significantly affect the function of PAK5.

The role of PAK5 in the regulation of actin cytoskeleton was further consolidated by its interaction with p120-Catenin (Wong, Reynolds et al. 2010). P120-Catenin is the founding member of the δ-Catenin family (Reynolds, Roesel et al. 1989), and is a well-characterised regulator of cytoskeletal reorganisation and cell-cell adhesion, which also has a feedback loop to the Rho GTPases. P120 interacts with the juxtamembrane domain of classical Cadherins, where it is essential for cadherin stabilisation (Reynolds, Herbert et al. 1992, Mo and Reynolds 1996). P120 is phosphorylated on multiple tyrosine, serine and threonine residues, but the distinct function of serine/threonine phosphorylation of p120 has yet to be elucidated (Xia, Mariner et al. 2003).

In the study on p120 interaction with group 2 PAKs (Wong, Reynolds et al. 2010), p120 was observed to preferentially interact with wild-type (WT) PAK5, constitutively-active PAK5 and constitutively-active PAK4, but not wt-PAK4, wt-PAK6 or constitutively active PAK6. Co-expression of PAK5 also increases the total protein level of p120 in the cells, in conjunction with elevated level of phosphorylation exclusively on serine 288 (S288), and it is hypothesised that serine phosphorylated p120 does not turn over as rapidly, and more stable in the cells in the

presence of PAK5 protein. The study also highlighted the distinct difference between PAK5 and oncogenic PAK4 in their interaction with a common substrate; p120-catenin, where PAK4 was demonstrated to phosphorylate additional serine/threonine sites, and have different subcellular co-localisation upon interaction with p120-catenin. Specific to the common interaction which involves phosphorylation of serine 288 of p120, PAK5 and phospho-S288 p120 colocalised in discrete vesicular structures in the cytoplasmic compartment, and neither PAK5 nor phospho-S288 p120 were detected in the nucleus. The co-localisation of constitutively active PAK4 and phospho S288 p120 was observed in diffuse cytoplasmic distribution, with marked nuclear localisation of p120.

P120-catenin is a tightly regulated protein, and in cancer studies, has been implicated to have a dual role in possessing both oncogenic and tumour suppressor properties (Schackmann, Tenhagen et al. 2013). It could therefore be postulated that PAK5 has a distinct function in the regulation of p120-Catenin, different to PAK4, which may facilitate or inhibit cancer progression. As the study was focussed neuronal cell models, the functional significance of the interaction between PAK5 and p120-Catenin in the epithelium, and the effect on cadherin-catenin adherens junction complex has not been elucidated. As PAK5 has no distinct transcriptional role, it may be necessary to utilise well differentiated human epithelial cell models, for which differentiated urothelial tumour cell lines may be suitable candidates.

In contrast to the well-studied tyrosine phosphorylation of p120 by growth-factor stimulation, the role for serine/threonine phosphorylation of p120 in Cadherin function is not very well understood. In the study characterising the interaction between PAK5 and p120-Catenin (Wong, Reynolds et al. 2010), PAK5 was shown to phosphorylate p120-catenin on Serine 288, but the effect of this interaction on the adherens junctions has not been assessed. However, a number of studies on cell lines with well-differentiated epithelial morphology have identified constitutive phosphorylation of serine/threonine residues in cells with functional E-Cadherin (Xia, Mariner et al. 2003, Xia, Carnahan et al. 2006), and over-expression of E-Cadherin in MiaPaCa-2 Cadherin-deficient cells resulted in serine/threonine phosphorylation of p120, which co-localised with E-cadherin to cell-cell junctions (Fukumoto, Shintani et al. 2008). Although the specific kinase involved was not identified in these studies, the authors had postulated that serine/threonine phosphorylation of p120 may provide initial signals during junction assembly, by either rapid organisation of E-

cadherin at cell-cell contact, by rescuing E-Cadherin from degradation and prolonging its localisation at the cell surface, or by providing a plasma retention signal.

The interactions previously described between both wild-type and constitutively active PAK5 with p120-Catenin, and the associated serine/threonine phosphorylation (Wong, Reynolds et al. 2010), suggest a potential role for PAK5 in the establishment of stable epithelial cell-cell adhesions. This postulation however, may be paradoxical to reports associating PAK5 expression with progression and metastasis of a number of epithelial cancers, including colorectal (Gong, An et al. 2009) and breast (Wang, Cheng et al. 2013) carcinomas. However, the mechanistic role of PAK5 in the progression of cancer described in these studies were mostly attributed to the anti-apoptotic or proliferative functions of PAK5, with limited information on cell invasion or migration.

Two further PAK5 substrates, Pacsin-1 and Synaptojanin-1 have recently been identified, which implicates PAK5 in synaptic vesicle trafficking (Strochlic, Concilio et al. 2012). Pacsin1 and Synaptojanin1 directly interact with one another to regulate synaptic vesicle endocytosis and recycling. Both proteins are phosphorylated by PAK5, and PAK5 phosphorylation promotes Pacsin1 and Synaptojanin1 binding both in *vitro* and in *vivo*. However, it is very unlikely that this interaction has a role in bladder cancer studies, as both Pacsin1 and Synaptojanin1 are highly brain specific in their expression, with absent or minimal expression in normal and malignant epithelial tissues (Ramjaun and McPherson 1996, Liu, Lv et al. 2012, Quan and Robinson 2013).

1.3.3 PAK5 in development

The specific functions of the group 2 PAK family members have been studied using gene knockout mice. PAK4 knockout mice die in utero, and the embryos have several structural abnormalities (Qu, Li et al. 2003). In contrast, PAK5, PAK6 and PAK5/PAK6-double knockout mice were viable and fertile, and it was postulated that PAK5 expression begins later in development and not essential for early embryonic development.(Li and Minden 2003, Nekrasova, Jobes et al. 2008, Furnari, Jobes et al. 2013). Initial study on PAK5 showed that in PAK5-null mice, the nervous system and other tissues in which PAK5 was normally expressed were structurally normal, which suggested functional redundancy between PAK5 and other Rho-GTPase targets (Li and Minden 2003). The hypothesis was further supported by findings that PAK5/PAK6 double knockout mice had several locomotor and behavioural deficits, compared to single PAK5 or single PAK6 knockout mice (Nekrasova, Jobes et al. 2008).

However, in the most recent study, PAK6 knockout mice were found to weigh significantly more than PAK5 or PAK5/PAK6 double knockout, and the PAK5 or PAK5/PAK6 double knockout mice performed worse on the rotorod test of motor co-ordination and balance, indicating distinct behavioural differences in the single knockout PAK5 and PAK6 mice (Furnari, Jobes et al. 2013).

1.3.4 PAK5 and Cancer

To date, PAK5 has not been shown to be involved in bladder cancer. However, evidence for PAK5's role in oncogenic pathways are emerging. PAK5 has been reported to be over-expressed in a variety of colorectal cancer cell lines, and immunohistochemistry of colorectal tumour resection specimens also showed higher expression level of PAK5 in poorly differentiated, invasive and metastatic colorectal tumours (Gong, An et al. 2009). PAK5 has also been demonstrated to inhibit camptothecin-induced apoptosis in colorectal carcinoma cells (Wang, Gong et al. 2010). In the study by Wang et al, camptothecin-induced activation of caspace-8 and PARP was shown to be inhibited in PAK5 overexpressing cells, whereas shRNA knockdown of PAK5 increased apoptosis in colorectal cells in response to camptothecin stimulation.

PAK5 has also been implicated in the carcinogenesis of gastric cancer (Gu, Li et al. 2013), where immunohistochemical evaluation of 57 tumour tissues and the corresponding adjacent normal gastric tissue detected significant PAK5 upregulation in gastric tumours, compared to normal adjacent tissues. However, it is interesting to note that upregulation of PAK5 in gastric cancer in the study did not correlate with histological differentiation, tumour size, TNM stage, lymphovascular invasion or distant metastasis. Further evaluation for the role of PAK5 by Lentivirus-mediated PAK5 siRNA knockdown was performed by Gu *et al*, and indicated that PAK5 has a significant role in gastric cancer cell growth, proliferation and cell-cycle regulation. In hepatocellular carcinoma, elevated mRNA and protein expression of PAK5 have also been detected in tumours, compared to the adjacent normal liver tissues (Fang, Jiang et al. 2014). In the study, Fang et al also demonstrated that PAK5 siRNA knockdown inhibited hepatocellular carcinoma cell proliferation in vitro, and tumour formation in nude mice following subcutaneous injection.

A study using breast cancer cell lines (MDA-MB-231 and BT549) had also implicated PAK5 in the migration and invasion of breast carcinoma through PAK5-Egr1-MMP2 signalling (Wang, Cheng et al. 2013).This study found that silencing of PAK5 inhibited breast cancer cell line proliferation, promoted apoptosis, and decreased cell migration in wound-healing and Matrigel transwell invasion assay. Knockdown of PAK5 in MDA-MB-231 and BT549 was associated with increased protein level of Egr1, and decreased level of cleaved MMP2. MMP2 is one of the proteases

involved in breast cancer invasion, associated with poor prognosis (Talvensaari-Mattila, Paakko et al. 2001, Talvensaari-Mattila, Paakko et al. 2003), and Egr1 is a member of the immediate gene family of transcription factors which can bind to MMP2 promoter and inhibit its activity (Zcharia, Atzmon et al. 2012). In epithelial ovarian cancer (EOC), the expression of PAK5 was associated with the occurrence and development of EOC, and PAK5 overexpression may also promote paclitaxel-chemoresistance (Li, Yao et al. 2013). Using immunohistochemistry, Li et al found that PAK5 was overexpressed in epithelial ovarian cancer, and the increase of PAK5 expression was also associated with EOC progression through the adenoma to carcinoma sequence, with the highest expression level in invasive and metastatic EOC. In *vitro studies* by this group demonstrated that in SK-OV-3 cells, downregulation of PAK5 by shRNA significantly affected proliferation and apoptosis upon treatment with paclitaxel as chemotherapeutic agent.

Systematic sequencing of cancer genomes had also identified PAK7 gene (which encodes for PAK5 protein) as one of the top 20 (positioned 17th out of 20) protein kinase genes ranked by probability to be carrying driver mutations in several cancers which include breast, lung, gastric, renal and ovarian carcinomas (Greenman, Stephens et al. 2007). Despite the large scale study carried out by Greenman *et al*, the data on whether somatic mutation in bladder cancer was unclear, as bladder cancer tissues were not screened in this study.

Complimentary to the study by Greenman *et al*, targeted genetic dependency screen has facilitated identification of actionable mutation in FGFR4, MAP3K9, and PAK7 in lung cancer (Fawdar, Trotter et al. 2013). The gain of function (GOF) mutations in these three different kinases were activating towards the ERK pathway, and the study demonstrated that targeted depletion of the kinases with siRNA resulted in reduced viability of lung cancer cells. Specific to PAK5, lung cancer H2087 cell line was used by Fawdar et al as a model. H2087 cell line harbours 79 non synonymous somatic mutations in 79 different genes, among 4700 genes sequenced (Greenman, Stephens et al. 2007). Using on-target siRNA SMART pools, the transcripts that carried the 79 nonsynonymous mutations were depleted and the effects on proliferation were monitored. Based on the screening criteria, PAK5 was found to have the most significant Strictly Standardised Mean Difference (SSMD) value for H2087 cell line proliferation, and the study further verified this with Caspace assay. The single PAK5 siRNA oligos was demonstrated to significantly suppressed proliferation, induced apoptosis and attenuated signalling in the in the MAPK/ERK pathway.

1.3.5 PAKs and urothelial carcinoma

PAK1 is the most extensively studied member of the PAK family and has been studied in the context of urothelial carcinoma. Increased Rac1 activity and PAK1 over-expression in urothelial carcinoma of the upper urinary tract were associated with lymphovascular invasion and lymph node metastasis based on analyses of surgical specimen(Kamai, Shirataki et al. 2010). Oligo-microarray of surgical specimen from transurethral resection of bladder tumour (TURBT) also identified PAK1 to be associated with increased risk of intravesical recurrence, independent of histopathological grade and stage(Ito, Nishiyama et al. 2007). Wound healing assay was performed in bladder cancer 253J cells transfected with constitutionally active T234E-PAK1, and EJ cells transfected with kinase dead K299R-PAK1(Ito, Nishiyama et al. 2007). The cells with constitutionally active PAK1 completed the wound healing process, whereas the kinase dead form did not migrate.

The oncogenic PAK1 in bladder cancer has recently been reported to be modulated by tumour suppressor microRNA; miR-145 (Kou, Gao et al. 2014). The level of miR-145 negatively correlates with PAK1 protein expression in bladder cancer, and miR-145 directly targets PAK1 to inhibit bladder cancer cell invasion partly by suppressing the protein expression of PAK1 and one of its downstream effectors; metallic metalloproteinase 9 (MMP9).

Paradoxically, a recent study has demonstrated that PAK1 may be a marker of treatment response of bladder tumours to BCG therapy (Redelman-Sidi, Iyer et al. 2013). BCG intravesical therapy is an established adjuvant treatment for high risk NMIBC, which reduces the rate of recurrence of NMIBC (Sylvester, van der et al. 2002, Babjuk, Burger et al. 2013). Despite being one of the most successful cancer immunotherapy, its mechanism of action and response determinants remains obscure. The study published by Redelman-Sidi et al demonstrated that the uptake of BCG into bladder cancer cells occur by macropinocytosis, and this process relies upon Rac1, Cdc42, and one of its effectors, PAK1 and to a lesser extent PAK2. The study however did not specifically tested the role of group-2 PAKs in the uptake of BCG into bladder cancer cells. PAK1-dependent macropinocytosis of BCG into bladder cancer cells was independent of Dynamin and Clathrin, and the internalised BCG colocalised with fluid-phase fluorescent dextran (MW 10000).

Despite these findings, PAK1 has not yet been integrated into the clinical management of bladder cancer as a molecular biomarker of disease progression, treatment response, nor therapeutic target. Apart from PAK1, the role of other members of the PAK family in bladder cancer has not yet been characterised. As members of group 1 PAKs have been more extensively studied, I have decided to focus my research on the group 2 PAKs expression in bladder cancer.

Aims

Despite their structural similarities, PAK4, PAK5 and PAK6 appear to have distinct roles in oncogenesis. In studying bladder cancer, it would be pertinent to further assess the role of these novel member of the PAK family in the loss of epithelial morphology in bladder tumour cells, indicating epithelial to mesenchymal transition associated with invasion and metastasis of bladder carcinoma.

I therefore aimed to identify and characterise additional member(s) of the PAK family with distinct role in regulation the activation or suppression of invasion and metastasis in bladder cancer. PAKs may play a role in one or more steps in the complex cascade of metastasis, which include altered adhesion (invasion), intravasation, survival in the circulation, extravasation and seeding at distant metastatic site (metastatic colonisation) (Talmadge and Fidler 2010).

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 General materials

Reagent	Supplier
Accuprime PFX supermix	Invitrogen, UK
Acrylamide (30%)	Severn Biotech Ltd, UK
Agarose	Invitrogen, UK
Ammonium persulfate (APS)	Sigma Aldrich, UK
Ampicillin	Sigma Aldrich, UK
Aprotinin	Sigma Aldrich, UK
Beta (β) mercaptoethanol	Sigma Aldrich, UK
Bovine serum albumin (BSA)	VWR International, UK
Bromophenol blue	Bio-Rad, UK
Calcium phosphate transfection kit	Invitrogen, UK
Carbenicillin	Sigma Aldrich, UK
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, UK
Dithiothreitol (DTT)	Sigma Aldrich, UK
DNA (100bp) DNA ladder	New England Biolabs, UK
DNA (1kb) DNA ladder	New England Biolabs, UK
Dulbecco's Modified Eagle's Medium (DMEM)	GIBCO®, Invitrogen, UK
Dulbecco's phosphate-buffered saline (DPBS)	GIBCO®, Invitrogen, UK
Epidermal growth factor (EGF), (Recombinant Human)	R&D systems, USA
Enhanced chemiluminescense (ECL) Western blotting detection system	Amersham Biosciences, UK
Ethidium Bromide	Thermo Fisher Scientific, UK
Ethylene diamine tetraacetic acid (EDTA)	Sigma Aldrich, UK

FluorSave™ reagent	Calbiochem, UK
Foetal Bovine Serum (FBS)	GIBCO®, Invitrogen, UK
Gateway™ BP Clonase™ 11 Enzyme mix	Invitrogen, UK
Gateway™ LR Clonase™ 11 Enzyme mix	Invitrogen, UK
Gentamicin	Sigma Aldrich, UK
Glutaraldehyde	Sigma Aldrich, UK
Glycerol	Sigma Aldrich, UK
Glycine	Sigma Aldrich, UK
Hepes, acid free, ULTROL grade	Calbiochem, UK
Hepatocyte Growth Factor (HGF), human recombinant	R&D systems, USA
Kanamycin	Invitrogen, UK
Leupeptin	Sigma Aldrich, UK
Lipofectamine® RNAiMAX	Invitrogen, UK
Luria-agar (L-agar)	Sigma Aldrich, UK
Luria-broth (LB)	Sigma Aldrich, UK
MAX Efficiency® DH5α™ Competent cells	Invitrogen, UK
NEB-10-Beta Competent *E. coli* cells	NEB, UK
Nitrocellulose membrane	Perkin Elmer, USA
Octylphenoxylpolyethoxyethanol/Nonidet™ P40 (NP40) substitute	Sigma Aldrich, UK
One Shot® TOP10 chemically competent E.coli	Invitrogen, UK
OptiMEM	GIBCO®, Invitrogen, UK
PAK7 siRNA oligonucleotides	*See oligonucleotide list*
Paraformaldehyde (PFA)	Sigma Aldrich, UK
Penicillin-streptomicin (100x)	Sigma Aldrich, UK
Phenylmethysulfonylfluoride (PMSF)	Sigma Aldrich, UK
Phosphate buffered saline (PBS) tablets	Oxoid Ltd, UK
Pierce® ECL Western Blotting substrate	Thermo Scientific, USA
Protein A Sepharose™ fast flow beads	Invitrogen, UK

Protein G Sepharose™ fast flow beads	Sigma Aldrich, UK
Precision Plus Protein™ all blue standards	Bio-Rad, USA
Purelink™ Hi Pure Plasmid Miniprep kit	Invitrogen, UK
Purelink™ Hi Pure Plasmid Maxiprep kit	Invitrogen, UK
Q5® Hot Start High-fidelity DNA polymerase	NEB, UK
Qiaprep® spin Miniprep kits	Qiagen Ltd, UK
QIAquick gel extraction kit	Qiagen Ltd, UK
RNeasy Mini Kit for RNA purification	Qiagen Ltd, UK
RPMI Media 1640	GIBCO®, Invitrogen, UK
Sodium chloride (NaCl)	Sigma Aldrich, UK
Sodium dodecyl sulphate (SDS)	Sigma Aldrich, UK
Sodium fluoride (NaF)	Alfa Aesar, UK
Sodium orthovanadate (Na_3VO_4)	Sigma Aldrich, UK
Sodium pyrophosphate	BDH Chemicals, UK
Sucrose	Sigma Aldrich, UK
SulfoLink Immobilization Kits and Coupling Resin	Thermo Scientific, USA
Tris (Tris(hydroxymethyl)aminomethane) - base	Sigma Aldrich, UK
Triton X-100	VWR International, UK
Trypsin/EDTA	GIBCO®, Invitrogen, UK
Tween 20	VWR International, UK
X-ray films	Scientific Laboratory Supplies, UK
X-tremeGENE HP DNA transfection reagent	Roche, UK

Table 2-1 : List of general materials and reagents

2.1.2 **Mammalian cell lines**

Bladder cancer cell lines: RT4, RT112, T24, TCCSUP and 253J were gifts from Professor John Masters, University College London. The origin of these cell lines are listed in table (***x)*** (Masters, Hepburn et al. 1986). Cell line authentication by STR analysis was performed on these cell lines by our collaborator and no cross contamination by another human cell lines were detected (appendix 1).

Cell line	Tumour origin	Clinical stage	Histological grade	Sex of patient
T24	Primary bladder	Not recorded	High grade	Female
RT112	Primary bladder	Stage 2	Low grade	Male
RT4	Primary bladder	Not recorded	Low grade	Male
TCCSUP	Primary bladder	Stage 4	High grade	Female
253J	Retroperitoneal lymph node metastasis	Stage 4	High grade	Female

Table 2-2 : List of bladder cancer cell lines used in the study

Human embryonic kidney 293 (HEK293) cells line was obtained from Dr Claire Wells, King's College London. Cells were cultured in DMEM supplemented with glucose (4500mg/L), L-glutamine, NaHCO3, pyridoxine HCl, 10% foetal bovine serum and 1% penicillin/streptomycin (100U/ml penicillin and 100μg/ml streptomycin). HEK293 cells were incubated in the same condition as the bladder cancer cell lines.

2.1.3 **Plasmids and Vectors**

Plasmid/Vector	Supplier/Source
PAK5 cDNA (ORF)	Thermo Scientific Open Biosystems Human ORFeome Collaboration Clone
PAK5 (Wild type) Gateway™ Entry Clone	Generated using Gateway™ Technology Cloning system
PAK5 (N-terminal) Gateway™ Entry Clone	Generated using Gateway™ Technology Cloning system
PAK5 (C-terminal) Gateway™ Entry Clone	Generated using Gateway™ Technology Cloning system
pDONR™ 207	Invitrogen, UK
pEGFP-C1 Expression vector	Clontech, UK modified for use as a Gateway™ Technology Destination vector by Kerry Shea, KCL
pDEST™ monomeric Red Fluorescent Protein (mRFP) Expression vector	Invitrogen, UK modified for use as a Gateway™ Technology Destination vector by Kerry Shea, KCL
pDest™ myc (pRK5-myc) Expression vector	Clontech UK modified for use as a Gateway™ Technology Destination vector by Kerry Shea, KCL
GFP-Cdc42 (wild type)	A generous gift from Dr Maddy Parsons, KCL
E-cadherin GFP	A generous gift from Dr Penny Morton, KCL
GFP-PAK5	Generated using Gateway™ Technology Cloning system
mRFP-PAK5	Generated using Gateway™ Technology Cloning system
Myc-PAK5	Generated using Gateway™ Technology Cloning system
Myc-NPAK5	Generated using Gateway™ Technology Cloning system

Myc-CPAK5	Generated using Gateway™ Technology Cloning system
GFP-PAK4	A generous gift from Dr Anna Dart, KCL
GFP-PAK6	A generous gift from Dr Sally Fram, KCL
P120 (wild type) VSV	A generous gift from Professor Anne Ridley, KCL
P120 S288A VSV	A generous gift from Professor Anne Ridley, KCL

Table 2-3 : List of plasmids used in the study

2.1.4 **Small interfering RNA (siRNA)**

SiRNA	Supplier	siRNA Data	
Control siRNA	Qiagen (1022076)	Sense Sequence	UUCUCCGAACGUGUCACGUdTdT
		Antisense Sequence	ACGUGACACGUUCGGAGAAdTdT
		Target DNA Sequence	AATTCTCCGAACGTGTCACGT
PAK5 siRNA-70	Qiagen (SI02225370)	Sense sequence	GGUGUGCACGUUUCAUUAATT
		Antisense sequence	UUAAUGAAACGUGCACACCAT
		Target DNA sequence	ATGGTGTGCACGTTTCATTAA
PAK5 siRNA-63	Qiagen (SI02225363)	Sense sequence	GAUCUGGAUCCGUAUUAUATT
		Antisense sequence	UAUAAUACGGAUCCAGAUCAT
		Target DNA sequence	ATGATCTGGATCCGTATTATA

Table 2-4 : Small interfering RNAs used for PAK5 (PAK7 gene) knockdown experiments

2.1.5 Primers

All Primers were ordered from Thermo Fisher Scientific, Germany

2.1.5.1 Primers for cloning

Target		Sequence 5' --→ 3'	Product size, bp	
Full length PAK5	F	GGGG ACA AGT TTG TAC AAA GCA GGC TTG ATG TTT GGG AAG AAA AAG AAA	2160	Gateway™
	R	GGG A CCA CTT TGT ACA AGA AAG CTG GGT C TCA GTG ATG CCT GTA TTG TCT		
N-terminal PAK5	F	GGGG ACA AGT TTG TAC AAA GCA GGC TTG ATG TTT GGG AAG AAA AAG AAA	1275	Gateway™
	R	GGGG A CCA CTT TGT ACA AGA AAG CTG GGT C GGA GGG CTG GTC GGA GCC CCA		
C-terminal PAK5	F	GGGG ACAC AGT TTGTAC AAA GCA GGC TTG AGG GTG TCC CAT GAA CAG TTT CGG	885	Gateway™
	R	GGG A CCA CTT TGT ACA AGA AAG CTG GGT C TCA GTG ATG CCT GTA TTG TCT		
N-terminal PAK5	F	AACAAA GAATTC ATG TTT GGG AAG AAA AAG AAA AAG ATT G	1298	EcoR1
	R	AACAAA GCGGCCGC CTA CGA AAC TGT TCA TGG GAC AC		Not1
C-terminal PAK5	F	AACAAA GAATTC AGG GTG TCC CAT GAA CAG TTT CGG	884	EcoR1
	R	AACAAA GCGGCCGC TCA GTG ATG CCT GTA TTG TCT CAT GAG G		Not1

Table 2-5 : Primers used for cloning full length, N-terminal domain and C-terminal domain of PAK5

2.1.6 **Primers for Quantitative RT-PCR**

Primers were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The reverse primers were incorporated with Z-motif (highlighted in red), a sequence complimentary to the Ampliflor Uniprimer probe (Intergen Inc., New York, NY).

Target	Sequence		Product size, bp
GAPDH	F	GTGAAGGTCGGAGTCAACG	112
	R	ACTGAACCTGACCGTACA TGAGGTCAATGAAGGGGTC	
PAK1	F	CCAGTGATTGCTCCACGCCCA	102
	R	ACTGAACCTGACCGTACA GGGAGATGTAGCCACGTCCCGA	
PAK5	F	CCTCAGCCTCTCATCCAGCACC	99
	R	ACTGAACCTGACCGTACA AGGGCCGCCCGAAACTGTTC	
PAK6	F	ACAAGCAGATGCCGTGGCCC	109
	R	ACTGAACCTGACCGTACA CGCTGCGAGGCACCCTGAAA	

Table 2-6 : RT-qPCR primers used for quantification of PAK1, PAK5 and PAK6 mRNA in human bladder cancer cell lines and tissue samples

2.1.7 **Antibodies**

2.1.7.1 Primary antibody

Antibody	Host	Company	Dilution		
			WB	IF	IP
α-catenin	Rabbit	Sigma	1:1000		
Akt	Rabbit	Cell Signalling	1:500		
C-myc (9E10) #E3112	Mouse	SCBT	1:2000	1:200	1:400
α-tubulin	Mouse	Sigma	1:2000	1;400	
β-catenin	Rabbit	Sigma	1:1000		
β-tubulin	Mouse	Sigma	1:2000	1:400	
C-Met (C-12)	Rabbit	SCBT	1:1000		
E-Cadherin	Mouse	Zymed	1:1000	1:400	1:250
E-Cadherin	Mouse	ABCAM	1:1000	1:400	
EGFR #2232	Rabbit	Cell Signalling	1:1000		
EGFR #4267	Rabbit	Cell Signalling	1:1000		
ERK1/2 (MAPK)	Rabbit	Cell Signalling	1:1000		
GAPDH	Mouse	Millipore	1:20000		
GAPDH	Mouse	SCBT	1:20000		
GFP	Mouse	Roche	1:1000		1:250
HA (Y-11)	Rabbit	SCBT	1:1000		1:300
cMyc Ab	Mouse	SCBT	1:1000		
P120-catenin (pS288)	Mouse	BD Transduction	1:1000	1:200	
P120-catenin	Mouse	BD Transduction	1:2000	1:400	
PAK1	Rabbit	Cell Signalling	1:1000		1:250
PAK4 (#3242)	Rabbit	Cell Signalling	1:1000		
PAK4	Rabbit	Wells lab	1:2000		
PAK5	Rabbit	Abcam	1:500		
PAK5	Rabbit	Millipore/Calbiochem	1:500		
PAK5	Rabbit	Wells lab	1:2000		1:250

PAK5 (H-20)	Goat	SCBT	1:500		
PAK5 (S-16)	Goat	SCBT	1:500		
Phospho ERK1/2 (pMAPK)	Rabbit	Cell Signalling	1:1000		
Phospho-Akt (Ser473)		Cell Signalling	1:1000		
phospho-Pak1/2/3	Rabbit	Cell Signalling	1:500		
phospho-Pak4/5/6	Rabbit	Cell Signalling	1:500		
Red Fluorescent Protein (RFP)	Rabbit	Biovision	1:1000		1:250
Vinculin	Mouse	Sigma	1:1000		

Table 2-7 : List of antibodies used in the project, with the concentration for use in Western blots, indirect immunofluorescence and immunoprecipitation as indicated

2.1.7.2 Secondary antibody/miscellaneous

Antibody	Company	
Alexa Fluor® 488 goat anti rabbit IgG	Invitrogen, UK	1:400 (IF)
Alexa Fluor® 568 goat anti rabbit IgG	Invitrogen, UK	1:400 (IF)
Alexa Fluor® 488 goat anti mouse IgG	Invitrogen, UK	1:400 (IF)
Alexa Fluor® 568 goat anti mouse IgG	Invitrogen, UK	1:400 (IF)
Goat anti-mouse IgG-HRP	Dako	1:2000 (WB)
Goat anti-rabbit IgG-HRP	Dako	1:2000 (WB)
Rabbit anti-goat IgG-HRP	Dako	1:2000 (WB)
Rabbit anti-mouse IgG-HRP	Dako	1:2000 (WB)
Phalloidin, Alexa Fluor® 488	Invitrogen, UK	1:400 (IF)
Phalloidin, Rhodamine (Molecular probes®)	Invitrogen, UK	1:400 (IF)
DAPI		1:10000 (IF)

Table 2-8 : List of secondary antibodies used in the project, with the concentration for use in Western blots or indirect immunofluorescence as indicated

2.1.8 **Buffers**

Blocking solution for Western Blot: 5% w/v milk powder or 5% w/v Bovine Serum Albumin (BSA) in Tris buffered saline (TBS)-Tween

Blocking solution for Immunofluoresce: 3% BSA in Phosphate buffered saline (PBS)

DNA loading buffer: 40% w/v sucrose, 0.25% w/v bromophenol blue

NP-40 lysis buffer: 0.5% v/v NP-40, 30mM sodium pyrophosphate, 50 mM Tris-HCL pH 7.6, 150 mM NaCL, 0.1 mM EDTA and protease inhibitor cocktail

Protease inhibitor cocktail: 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM DTT

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) running buffer (10x): 250 mM Tris-base, 1.92 M glycine, 1% w/v SDS. Dilute to 1x with distilled water (dH_2O).

SDS-PAGE transfer buffer (10x): 250 mM Tris-base, 1.92 M glycine, Make up transfer buffer fresh on the day by diluting to 1x by adding methanol to a final concentration of 20% v/v.

SDS-PAGE sample buffer (2x): 100mM Tris-HCl pH 6.8, 4% w/v SDS, 20% w/v glycerol, 0.2% w/v bromophenol blue, 1:50 β-mercaptoethanol

Tris Acetate EDTA (TAE) buffer: 40mM Tris acetate, 1 mM EDTA

TBS-Tween: 25mM Tris HCl pH7.6, 50 mM NaCL, 0.1% v/v/ Tween20

PBS-Tween: PBS, 0.1% Tween20

Stripping buffer: 25 mM glycine pH 2, 1% w/v SDS

2.2 Methods

2.2.1 Mammalian cell culture

2.2.1.1 Bladder cancer cell lines

Bladder cancer cell lines RT4, RT112, T24, TCCSUP and 253J were cultured in RPMI-1640 medium supplemented with L-glutamine and $NaHCO_3$, 10% foetal bovine serum and 1% penicillin/streptomycin (100U/ml penicillin and 100μg/ml streptomycin). Cells were incubated at 37 ^{0}C in a humidified atmosphere of 5% CO_2 in air. All cells were confirmed to be mycoplasma-free by regular DAPI staining.

HEK293 cell line was cultured in DMEM with 10% FBS and 1% penicillin-streptomycin. All cell lines were cultured in tissue culture incubator with humidified air, supplemented with CO_2 to 5% above atmospheric level. Cells were passaged and maintained at subconfluent levels in T75 flasks. During cell-passage, the growth medium was removed and the cells were washed with 5 ml of PBS for 3 times prior to incubation with 2 ml Trypsin/EDTA at 37 ^{0}C for 5-10 minutes until the cells become detached from the surface.

2.2.1.2 Seeding cells on glass coverslips for morphological characterisation of cells in basal growth condition

Cells cultured in basal growth condition were seeded on glass coverslips for 24 hours to reach 50-60% confluency. Cells were then stained with Phalloidin to detect F-actin, and DAPI for the nucleus. The immunofluorescent images were analysed to assess the morphology, and the cell shape was quantified and analysed using ImageJ software.

2.2.1.3 Cryogenic storage of cell lines

Cells in T75 culture flask were trypsinised for 5-10 minutes until all become detached, and 4 mls of full growth medium was added. Cell suspension was then centrifuged at 1200rpm for 5 minutes, and the supernatant was discarded. Cells were resuspended in freezedown media (50% FBS, 10% DMSO and 40% RPMI-1640 or DMEM) and 1 ml of re-suspended cells were transferred into each cryovial. Cryovials were initially inserted into cryo-freezing container for slow-freezing overnight in the -80 ^{0}C before long-term storage in the liquid nitrogen tank.

2.2.1.4 Recovery of cells from cryogenic storage

Cells were recovered from long term storage in liquid nitrogen and thawed rapidly at 37 ^{0}c in a water bath. Thawed cell suspension was then transferred to a 15 ml tube, and 5ml of warmed, full growth medium was added slowly drop-wise. The cell suspension was then centrifuged at 1200rpm for 5 minutes, and the supernatant was discarded. The cell pellet was then resuspended in 5 mls of full growth medium, and transferred into a 25ml tissue culture flask (T25) for culture. The full growth media would be refreshed after 24 hours, and the cells were passaged at 80% subconfluent monolayer.

2.2.2 Microscopy

2.2.2.1 Immunofluorescent labelling

Cells on coverslips were fixed with 4% PFA in PBS at room temperature for 30 minutes and washed 3 times with PBS. The cells were then permeabilised with 0.2% Triton X-100: PBS for 5 minutes, and washed 3 times with PBS. The cells were then incubated in blocking buffer of 3% BSA in PBS for 30 minutes, and washed 3 times with PBS. Next, the cells were incubated with the required primary antibody for 2 hours, and washed 3 times with PBS. Following this step, the cells were incubated with Alexa Fluor® 488 or 568, (Mouse/Rabbit specificity) according to the species from which the primary antibodies were raised. If actin co-staining is required, Phalloidin of complementary fluorescent emission (Trit-C/Rhodamine/488/633) was added for incubation together with the secondary antibody solution. For nuclear staining, DAPI (1:10000 in PBS) was added following secondary antibody incubation and the coverslips were incubated for further 5 minutes. The cells on coverslips were then washed 2 times with PBS and once in ddH_2O before being mounted on glass slides using 10μL FluorSave™ reagent and left to dry overnight in dark environment. The coverslips on glass slides are kept in dark environment at 4 ^{0}c.

The cells on coverslips were imaged on an Olympus IX71 inverted microscope system or Nikon A1R Confocal Microscope system.

2.2.3 Molecular biology

2.2.3.1 Generation of PAK5 overexpression plasmid using Gateway™ Cloning Technology

Polymerase Chain Reaction (PCR) amplification of PAK5 cDNA

Polymerase chain reaction amplification of PAK5 cDNA was performed using attB tagged forward and reverse primer sequences using the Gateway™ Technology system (Invitrogen, UK). PAK5 wild-type plasmid was used as the DNA template in the production of PAK5 DNA flanked by *att*B sequences (the addition of *att*B sequences was required to allow for subsequent cloning into Gateway™ vectors). The PCR reaction was performed using Accuprime PFX Supermix DNA polymerase. For a 50μl reaction, the components listed in **table 2-4** were added to the reaction tube. The specific conditions used for the PCR reaction for DNA amplification are displayed in **table 2.5.**

Component	Volume
Accuprime PFX Supermix	45 μl
Forward primer (10pmol/μl)	2 μl
Reverse primer (10pmol/μl)	2 μl
Template DNA (1000ng/μl)	1 μl

Table 2-9 : PCR reaction components of PAK5 cDNA amplification

Cycle(s)	Process	Temperature	Duration
1	Pre-incubation	95ºC	5 minutes
35	Denaturation	95ºC	15 seconds
	Annealing	58ºC	30 seconds
	Extension	68ºC	2 minutes 30 seconds
1	Final Extension	68ºC	10 minutes

Table 2-10 : Conditions for PCR amplification of PAK5

Gel purification of DNA fragments

Agarose gel electrophoresis was performed to isolate PCR product of PAK5 fragment estimated to be at 2.1kb. PCR products were separated on a 1% w/v TAE agarose gel supplemented with 0.5 µg/ml ethidium bromide and the band of DNA was visualised under low intensity UV light. The fragment of interest (at 2.1kb) was excised from the gel with a fine scalpel and the DNA extracted from the agarose gel using QIAquick gel extraction kit. The purified DNA was eluted in the elution buffer and then stored at -20°C for subsequent use.

Construction of Entry Clone

In order to generate a PAK5 entry clone, a Gateway™ BP recombination reaction was conducted between the pDONR™ 207 vector and the *att*B sequence flanked PAK5 PCR product in accordance with the manufacturer's instructions. The BP reaction was incubated for 1 hour at room temperature. Subsequently, proteinase K was added and the reaction mixture was incubated at 37 ºC for 10 minutes to terminate the reaction. The BP reaction mixture was then transformed into TOP10 *E. coli* cells and the bacteria were plated onto L-agar supplemented with the appropriate antibiotic and incubated overnight at 37ºC. Colonies were then selected and the plasmid DNA was purified. The presence of PAK5 in individual colonies was verified by restriction digest. PAK5-positive clones were subsequently sequenced by Eurofins MWG Operon using primers that anneal to the gateway sequence upstream of the PAK5 gene An internal primer was also generated to sequence the entirety of PAK5 **.**

Construction of PAK5 expression clone

In order to generate GFP-, RFP-, myc-, and HA-PAK5 expression plasmids, Gateway™ LR recombination reactions were performed between the pENTR (PAK5) entry clone and the selected destination vectors including modified pEGFP-C1 (Clontech, UK), pDEST™ mRFP, and pDEST™ myc (pRK5-myc) in accordance with the manufacturer's instructions. The LR reaction was incubated for 1 hour at room temperature. Subsequently, proteinase K was added and the reaction mixture was incubated at 37 ºC for 10 minutes to terminate the reaction. The reaction was then transformed into TOP10 *E. coli* cells as described in previously. The plasmid DNA

purified from the individual colonies was then sequenced by Eurofins MWG Operon using sequencing primers.

2.2.4 Transient transfection of cell lines

Transient transfection of HEK293 cells using Calcium Phosphate Transfection Kit

HEK-293 cells were seeded at a density of 1 x 10^5 cells/ml in 2mls of full growth medium, into 2cm wells on a 6-well plate. The cells were incubated overnight, and the medium was changed to a fresh full-growth medium 3-4 hours prior to transfection. The composition of the transfection mixture are documented in **table 2.6**.

2cm dish (2ml)/ 6-well plate	
Tube A	6 μl 2.5M $CaCl_2$ 2 μg DNA Make reaction volume up to 60 μl with sterile water
Tube B	60 μl 2x HEPES buffered saline (HBS)

Table 2-11 : Calcium Phosphate transfection mixture for HEK-293 cells

Mixture from tube A was slowly added with aeration to tube B, and incubated for 30 minutes at room temperature. The transfection mixture was then added to the HEK-293 cells in the 2cm wells drop-wise, and dispersed for even spreading. The cells were incubated overnight with the transfection mixture at 37 ^{0}c in the tissue culture incubator, with a change of medium to fresh full-growth medium at 24 hours. The cells were incubated for further 24 hours before the cells were fixed for imaging or lysed for protein immunoblotting.

Transient transfection of bladder cancer cell lines using X-tremeGENE HP

Bladder cancer cell lines were seeded at the appropriate density in full growth RPMI medium, and incubated at 37 ^{0}C overnight to attain 60-70% sub-confluent monolayer prior to transfection. The DNA-lipid complex for transfection in 6-well plate was prepared as follows:

Reagent	Volume
Optimem	92 μl
cDNA (at 1 μg/μl)	2 μl
X-tremeGENE HP	6 μl

Table 2-12 : Transfection mix composition for X-tremeGENE HP

The total reaction volume in Optimem™ was 100 μl, with reagent to DNA ratio of 3:1. The mixture was incubated at room temperature for 20 minutes to allow complex formation. The RPMI growth media in the 6-well plates were replaced with serum free Optimem™ prior to adding the transfection mixture to the cells. The cells were then incubated at 37 ^{0}C for 6 hours, before replacing the Optimem™ medium with full-growth RPMI. The cells were incubated and checked for transfection efficiency at 24 and 48 hours.

Synthetic siRNA reverse transfection using Lipofectamine RNAiMax

RT112 cells and RT4 cells were reverse transfected using Lipofectamine™ RNAiMax. The protocol was adapted from the manufacturer's (Invitrogen™) instructions for Transfection of SiRNA into MCF7 (colony-forming breast cancer cell-line). Cells were transfected in 6-well and 24-well plate formats, with the reaction components as documented **table 2.13**.

Culture vessel	Volume of plating medium	Dilution medium for reverse transfection	RNAi (pmol)	Lipofectamine RNAiMax
24-well	500 μl	100 μl	2	1 μl
6-well	2500 μl	500 μl	10	5 μl

Table 2-13 : Reagent amounts and volumes for reverse transfection of RT112 and RT4 cells using Lipofectamine™ RNAiMax scaled to 6-well and 24-well plates.

2.2.5 Generation of PAK5 specific polyclonal antibody

Designing synthetic peptide for antigen recognition

Protscale software available on Expasy website http://web.expasy.org/protscale/ was used to identify the suitable peptide sequence on PAK5 for antigen recognition. Two different scales (Hopp & Woods, and Kyte & Doolittle) were used to assess the hydrophobicity and the hydrophilicity profiles of PAK5 sequence of amino acids. A synthetic peptide sequence (yreks lygdd ldpyy) corresponding to aa146-160 of PAK5 protein was identified as a suitable antigen, as represented in **figure 2.2**. The peptide identified was manufactured and inoculated into rabbit hosts for polyclonal antibody production by Eurogentec Ltd.

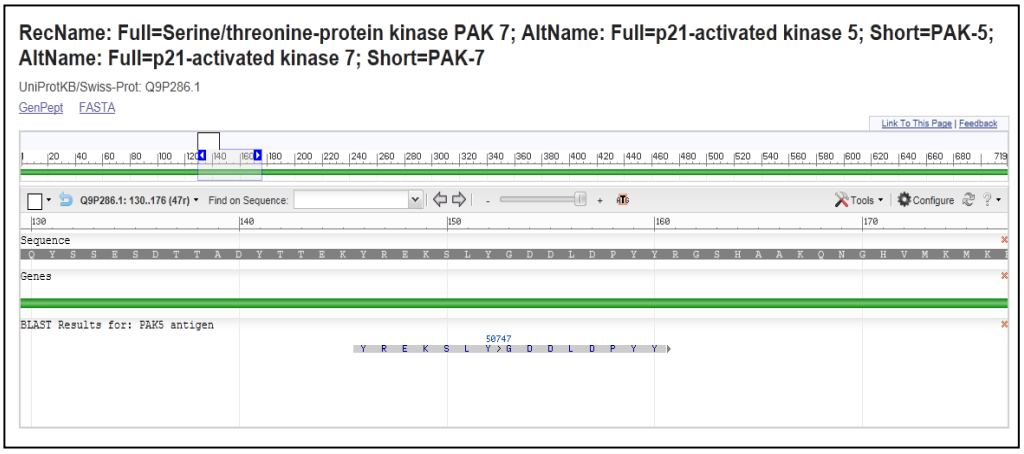


Figure 2-1 **:** The protein-protein blast for this peptide sequence showed that this epitope is PAK5 specific (http:/blast.ncbi.nlm.nih.gov).

2.2.5.1 Affinity purification of PAK5 rabbit polyclonal antibody

Serum containing PAK5-specific polyclonal antibody was then prepared for affinity purification using Sulfolink® Immobilisation kit for peptides according to manufacturer's protocol. Briefly, peptide column was prepared by coupling peptide sequence (yreks lygdd ldpyy) corresponding to aa146-160 of PAK5 to the resin within the column. Non-specific binding sites on the resin were blocked using 50 mM cysteine. The column with resin coupled to PAK5 peptide was then incubated with the serum of rabbit immunised with PAK5 for 45 minutes at room temperature to affinity purify the antibody. The column was then washed, and the antibody eluted.

2.2.6 Protein analysis

Cell lysis

Cells were washed twice in PBS and lysed on ice in 0.5% NP-40 lysis buffer supplemented with a protease inhibitor cocktail for 10 minutes. The whole cell lysates were then scraped and centrifuged for 10 minutes at 13000 x g for 10 minutes to remove cell debris. The supernatant was removed and placed in a fresh tube for storage. 10 µl of 2x SDS Gel sample buffer (GSB) was added to the lysates and the samples boiled at 90ºC for 3 minutes.

Immunoprecipitation

Typically 5 µl of the appropriate IP antibody was added to the lysis supernatant and incubated overnight at 4ºC on a rotating wheel. The following day, protein A or G beads were washed three times in lysis buffer. The beads were re-suspended in an appropriate volume of lysis buffer prior to adding 30 µl of bead slurry to each IP sample. The samples were then incubated for 1 hour on a rotating wheel at 4˚C. Each IP, and untransfected (UT) control, was pulse spun following incubation and washed three times with lysis buffer. 2x GSB was added to each sample and boiled at 90°C for 3 minutes.

Gel electrophoresis and immunoblotting

Proteins were loaded onto appropriate % gels and electrophoresed. Proteins were then blotted onto nitrocellulose membranes. The blots were then blocked in 5% milk or 5% BSA/TBS-Tween as appropriate for one hour at room temperature and incubated with primary antibodies overnight at 4°C. Blots were then washed for three 10 minute washes with 0.1% TBS-Tween. Blots were then incubated with HRP-conjugated secondary antibodies for one hour at room temperature. Blots were then subjected to a further three 10 minute washes with 0.1% TBS-Tween. If re-probing was required, the blots were incubated in stripping buffer for 15 minutes which was then removed and replaced with fresh stripping buffer for a further 15 minutes. The blots were then washed with 0.1% PBS-Tween for 5 minutes. Subsequently the blots were blocked for 1 hour in 5% milk or 5% BSA/TBS-Tween as appropriate and incubated overnight with primary antibodies at 4°C.

2.2.7 Data analysis

Image processing and cell shape analysis

Image J software was used to elucidate the morphology of each cell by manually drawing around individual cells and then processing the data to give elongation ratios and cell spread areas. In order to determine the elongation ratio of a cell, Image J shape analysis software divides the shortest cell diameter by the longest. Therefore, the ratio for an elongated cell morphology generated by Image J is a small numerical value. Hence, in order to aid graphical representation of the results acquired, all elongation ratio values have been subtracted from one and all spread areas have been multiplied by 1000.

Densitometry Analysis

The autoradiographs were saved as TIF files in Adobe Photoshop CS5 and ANDOR IQ Technology software was used to quantify desired protein levels. In this analysis system it was assumed that 0 is black and the maximal value is 255 at 8 bits per pixel. These values were then used to calculate the mean fold value.

Chapter 3 : Characterisation of bladder cancer cell lines and PAK expression

3.1 Introduction

P21-activated kinases (PAKs) are serine-threonine kinases that are positioned at the intersection of various signalling pathways required for oncogenesis (Molli, Li et al. 2009, Radu, Semenova et al. 2014). There is accumulating evidence that overexpression or aberrant activation of PAKs drives many of the cellular processes associated with biological capabilities that constitute the hallmarks of cancer, including activating invasion and metastasis (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011).

There are six mammalian PAKs, categorised into two subgroups on the basis of their sequence and structural homology. Group 1 PAKs comprises PAK1, PAK2 and PAK3, and the roles for PAK1 in cancer have been extensively characterised, including its role in the oncogenesis of urothelial tumours. In bladder cancer, overexpression of PAK1 was associated with increased risk of recurrence after transurethral tumour resection (Ito, Nishiyama et al. 2007), while in upper urinary tract urothelial tumours, overexpression of PAK1 was associated with lymphovascular invasion in tumour histopathology, and increased risk of metastasis on long term follow up (Kamai, Shirataki et al. 2010). The roles of other member of the PAK family in urothelial oncogenesis is unknown.

Group 2 PAKs consists of the more novel members; PAK4, PAK5 and PAK6. The best characterised member of this subgroup is PAK4. Amongst PAK4-6, PAK4 is most closely linked with cancer, and has been shown to promote anchorage independent growth, regulate cell cycle, inhibit apoptosis, and activate invasion and metastasis (Minden 2012, Dart and Wells 2013). Overexpression of PAK4 has been demonstrated in a variety of human cell lines and cancers, including gastric cancer (Ahn, Jang et al. 2011), ovarian cancer (Siu, Chan et al. 2010) and pancreatic cancer (Chen, Auletta et al. 2008). PAK5 has been shown to protect cells from apoptosis (Cotteret, Jaffer et al. 2003), and its overexpression has been associated with colorectal cancer (Gong, An et al. 2009) and gastric cancer (Gu, Li et al. 2013). PAK6, which was initially cloned from prostate cancer cells as an androgen receptor (AR) interacting protein(Yang, Li et al.

2001), has been shown to be involved in the regulation of prostate cancer (Wen, Li et al. 2009, Fram, King et al. 2013, Liu, Li et al. 2013). Despite the increasing interest in the roles PAKs in cancer, no studies have yet linked any of the group-2 PAKs to oncogenesis of urothelial carcinoma.

One of the aims of this study was to investigate the role of group 2 PAKs in the invasion and metastasis of bladder cancer. To initiate the project, 5 bladder cancer cell lines were identified to form a panel to screen the protein expression level of PAK4, PAK5 and PAK6. As PAK1 has previously been studied in bladder cancer, its expression level was also screened for its potential utility as an arbitrary positive control (Ito, Nishiyama et al. 2007, Kamai, Shirataki et al. 2010).

Many bladder cancer cell lines have been characterised and matched to represent clinical tumour stages and grades. The characterisation was based on their tumour of origin, cell morphology in culture, gene and protein expression, tumorigenicity and xenograft morphology (Masters, Hepburn et al. 1986, Ahlering, Dubeau et al. 1987, Theodorescu, Cornil et al. 1990, Rieger, Little et al. 1995, Hurst, Fiegler et al. 2004, Dancik, Ru et al. 2011). In this study, I started the project by using 5 different human bladder cancer cell lines; RT4, RT112, T24, TCCSUP and 253J.

The cell lines included in the panel were selected to represent urothelial cell lines at different stages of tumour progression, corresponding to well-differentiated, moderately-differentiated and poorly-differentiated primary urothelial tumour. A urothelial cell line (253J) from retroperitoneal lymph node with metastatic urothelial tumour was also included in the panel. The origins of these cancer cell lines are listed in table 3.1 (Rigby and Franks 1970, Bubenik, Baresova et al. 1973, Elliott, Cleveland et al. 1974, Nayak, O'Toole et al. 1977, Masters, Hepburn et al. 1986).

RT4 is a paradigm for well-differentiated bladder carcinoma. This cell line was established from primary bladder tumour (Rigby and Franks 1970), where the histological appearance of the original tissue cultured was of a well differentiated, low grade urothelial carcinoma of the bladder. RT112 is a moderately-differentiated urothelial carcinoma cell line which originated from a histology grade-2 (G2) papillary bladder tumour. In steady state, RT112 cells retain the epithelial-like morphology, in which the cells grow in distinct colonies to form an epithelial sheet in 2D culture (Mialhe, Levacher et al. 2000, Baumgart, Cohen et al. 2007).

Cell line	Tumour origin	Clinical stage	Histological grade	Sex of patient
T24	Primary bladder	Not recorded	High grade	Female
RT112	Primary bladder	Stage 2	Low grade	Male
RT4	Primary bladder	Not recorded	Low grade	Male
TCCSUP	Primary bladder	Stage 4	High grade	Female
253J	Retroperitoneal lymph node metastasis	Stage 4	High grade	Male

Table 3.1: Origin of bladder cancer cell lines used in the project

Tumours produced by RT4 and RT112 cell lines in nude mice xenograph models were shown to be morphologically conserved when the histopathological sections were compared to the original histopathological images taken in 1967 and 1973 respectively, despite years of culture and propagation (Masters, Hepburn et al. 1986). RT4 and RT112 had been used in many bladder cancer studies as the cell lines to represent low grade bladder tumours (Masters, Hepburn et al. 1986, Schwartz, Redwood et al. 1990, Theodorescu, Cornil et al. 1990, Weidner, Behrens et al. 1990, Bryan, Atherfold et al. 2008, Rose, Grandoch et al. 2010). Gene expression profiles of RT4 and RT112 cells have also been analysed, which showed molecular or gene expression alignment of these cells to low grade and low stage tumours when independently assessed in multi-centre cohorts (Dancik, Ru et al. 2011).

T24 and TCCSUP cell lines both originated from poorly differentiated, high grade primary urothelial tumours (Bubenik, Baresova et al. 1973, Elliott, Cleveland et al. 1974, Nayak, O'Toole et al. 1977). T24 was used in the study which identified the first human oncogene, H-ras (Taparowsky, Suard et al. 1982), which had led to significant advancement in cancer studies. The cell line 253J originated from metastatic retroperitoneal lymph node of a male patient with high grade (historically grade-4) bladder tumour with lymph node, bone and cerebral metastasis (Elliott, Cleveland et al. 1974, Nayak, O'Toole et al. 1977). It was interesting to note that the cell culture from this patient (253J donor) could only be established from the tissue taken from the retroperitoneal lymph node, but not the primary tumour, which indicated that tumour cells in metastasis may be characteristically different to the primary tumour cells in the same patient.

TCCSUP T24, TCCSUP and 253J have been used extensively in the study of bladder cancer, particularly as models to represent high grade bladder tumours (Steele, Rowlatt et al. 1983, Kwon, Yoshida et al. 1995, Bui, O'Brien et al. 1998, Pervaiz, Cao et al. 2001, Wu, Shu et al. 2003, Gallagher, O'Shea et al. 2008, Mariotti, Castiglioni et al. 2009, Moissoglu, McRoberts et al. 2009, da Silva, Evangelista et al. 2011, Mierke, Frey et al. 2011, Yamamoto, Sutoh et al. 2011). In this study, T24, 253J and TCCSUP were selected as the likely representatives of high grade, high stage, and invasive bladder cancer based on characterisation data already available in publications.

Cross contamination of cell lines is a well-documented problem in urology research (O'Toole, Povey et al. 1983, Masters 2002, Jager, Horiguchi et al. 2013). As these different cell lines were co-cultured at the same time in my project, the risk was significantly increased. A year after my project started, a collaborator had obtained the 5 bladder cancer cell lines from my stock and performed Short Tandem Repeat (STR) analysis. The analysis authenticated the cell lines obtained from my stock, and ruled out cross-contamination (appendix 1).

A number of discrepancies in the morphological characteristics of some of the cell lines in my panel have been reported when cultured in different laboratories (Mialhe, Levacher et al. 2000, Baumgart, Cohen et al. 2007). Thus, in this chapter, I first established the morphological characteristics of all the cell lines. I subsequently screened for expression of 2 classical markers of epithelial differentiation or EMT (N- and E-cadherin), and characterised the cadherin-based cell-cell adhesions in 2 of the cell lines. Finally, the bladder cancer cell-lines were screened for protein expression of PAK1 (positive control), and all group-2 PAK isoforms (PAK4-6).

3.2 Results

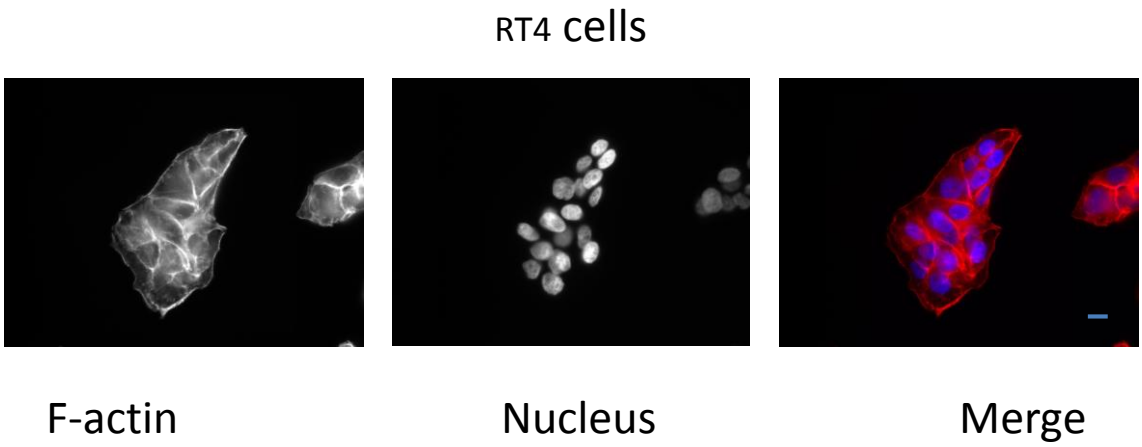
3.2.1 Cellular morphology of bladder cancer cell lines in culture

The actin cytoskeleton provides a structural framework around which cell shape and polarity are defined (Ballestrem, Wehrle-Haller et al. 1998, Hall 1998, Vasioukhin, Bauer et al. 2000). The morphology of bladder cancer cell lines were characterised by immunofluorescent staining of the F-actin which forms the cell cytoskeleton. Classification of epithelial morphology was broadly defined as tightly adherent cuboidal cells growing in discrete colonies, while mesenchymal morphology was defined as poorly adherent carcinoma cells displaying stellate morphology, as previously described (Baumgart, Cohen et al. 2007)

RT112 and RT4 cells both grew in distinct colonies which can form an epithelial sheet in 2-D, where the archetypal epithelial cell-cell junctions were conserved. Accumulation of F-actin at the margins of cell-cell contact was prominent in these cell lines (figure 3.1), which is also a phenotype commonly associated with epithelial cells (Vasioukhin, Bauer et al. 2000). Actin-based membrane protrusions such as filopodia or lamellapodia were rarely observed in RT4 cells when cultured in basal growth condition (figure 3.1A)

T24, TCCSUP and 253J cells, which were all derived from high grade/stage tumours, grow as detached cells in 2-D culture (figure 3.2) at 50-60% confluency. Distinct formation of cell: cell adhesion characterised by accumulation of F-actin at cell contact margins was not seen in these cells lines, even when the margins of adjacent cell were in contact with another (figure 3.2, indicated by arrows) Formation of thin, elongated membrane protrusions were seen in all 3 cell lines in this subgroup. These protrusions may represent filopodia, which are exploratory extensions from the plasma membrane that contain bundles of actin filaments, with important roles in both processes of cell migration (Mattila and Lappalainen 2008, Ridley 2011), as well as formation of cell-cell adhesions (Vasioukhin, Bauer et al. 2000).

A



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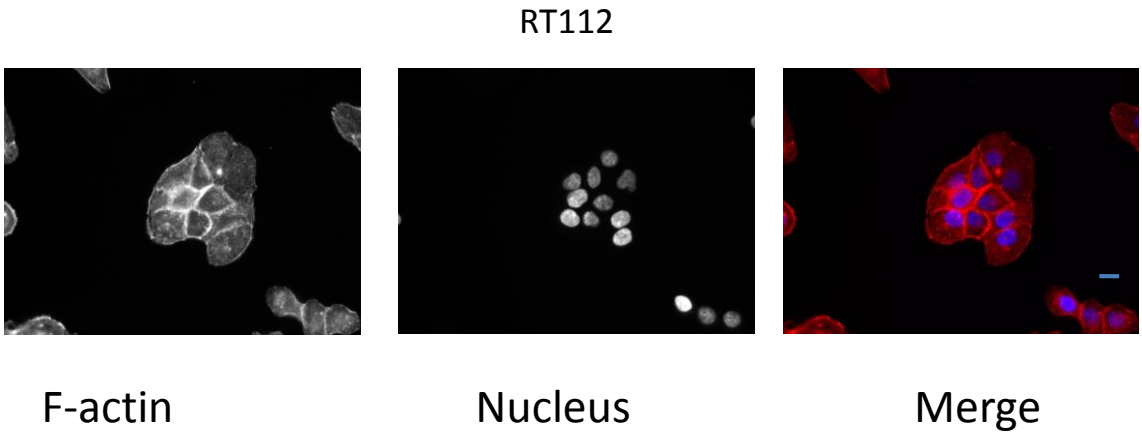
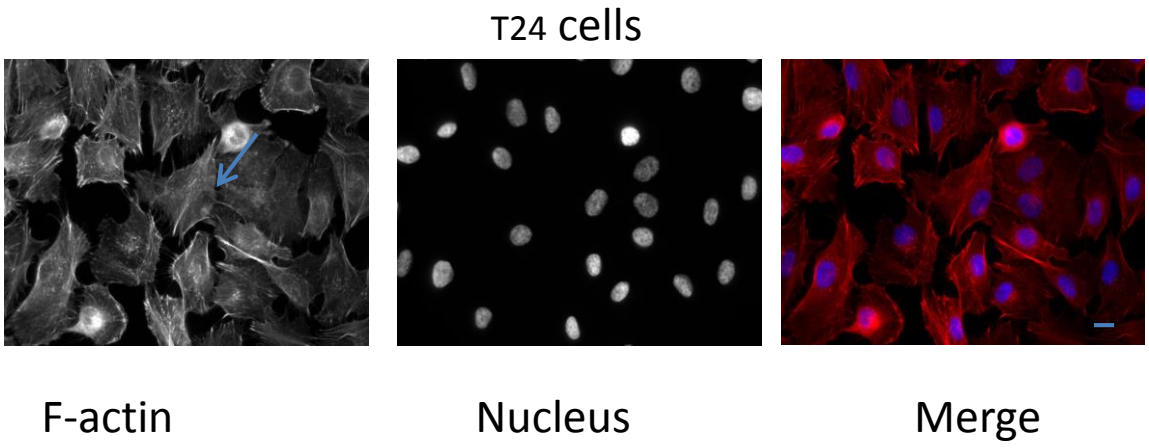
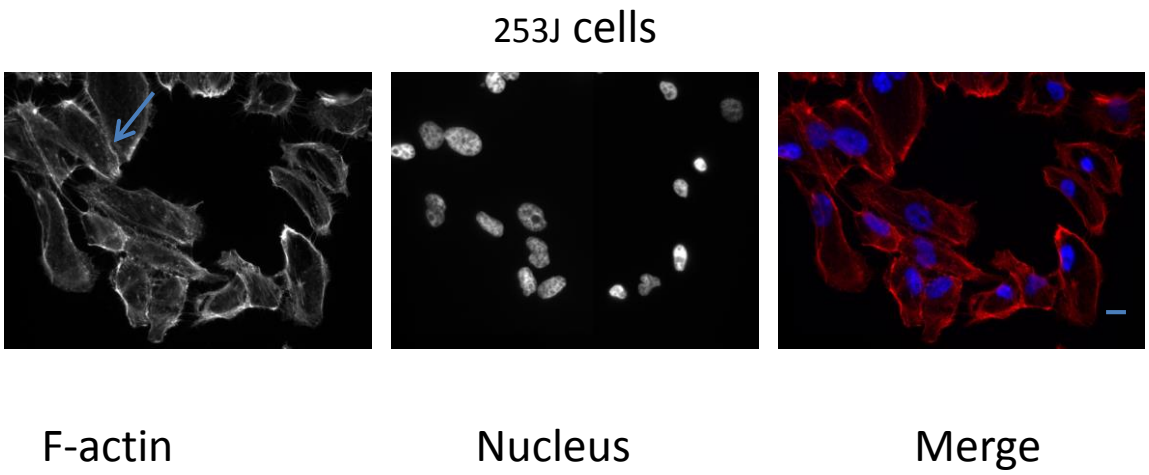


Figure 3-1 : **Morphological characteristics of well and moderately differentiated bladder cancer cell lines in 2D culture:** Immunofluorescent F-actin and nuclear staining of bladder cancer cell lines RT4 (A) and RT112 (B) in 40-60% subconfluent monolayer. Scale bar = 10μm.

A



B



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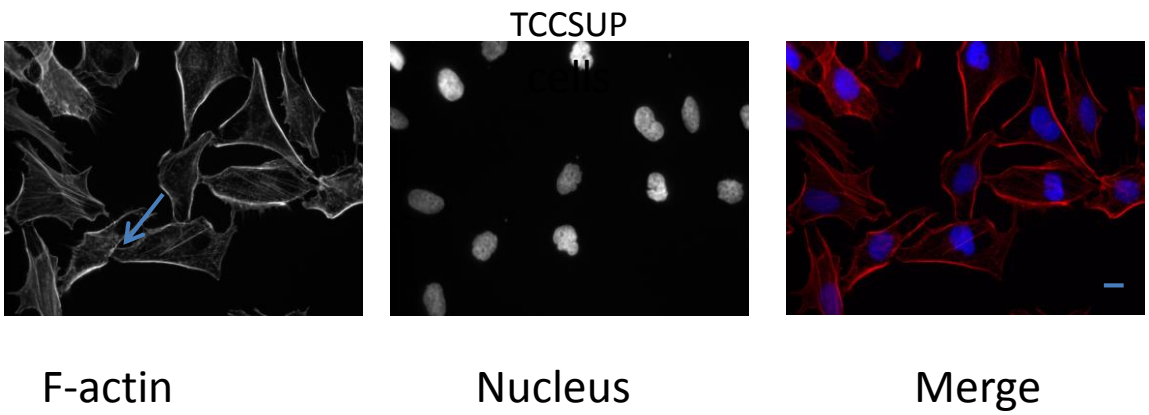
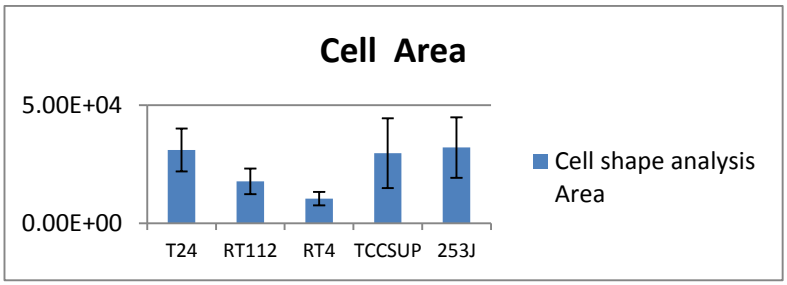


Figure 3-2 : **Morphological characteristics of poorly differentiated bladder cancer cell lines in 2D culture:** Immunofluorescent phalloidin staining for F-actin in T24 **(A)**, 253J **(B)** and TCCSUP **(C)** cells at 40-60% subconfluent monolayer. Arrows indicate areas of contact between adjacent cells without distinct accumulation of F-actin. Scale bar = 10μm.

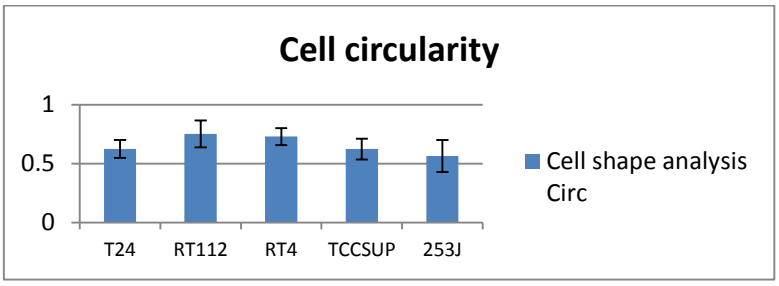
Cell shape in 2-D as indicated by the actin staining of the cell membrane, was analysed using imageJ software using set measurements, which included of area (relative value, 3.3A) and shape descriptors (circularity and aspect ratio, 3.3B-C). The analysis showed that RT112 and RT4 cells were smaller compared to T24, TCCSUP and 253J. The formation of cell-cell adhesions in the epithelial cells could significantly influence the cell shape observed at basal condition (Carthew 2005). Larger cell area on 2D in cell lines of invasive origin indicated greater cell spreading and motility potentials in these cell lines (Fardin, Rossier et al. 2010). TCCSUP cells had distinctly elongated morphology in basal growth condition, consistent with its mesenchymal phenotype.

The differences in cell morphology seen in this section (figures 3.1 and 3.2), suggested that high grade/stage bladder tumour cells (T24, TCCSUP and 253J) had lost their epithelial morphology and undergone epithelial to mesenchymal transition, whereas RT4 and RT112 cells still retained their epithelial morphology when cultured in basal growth condition.

A



B



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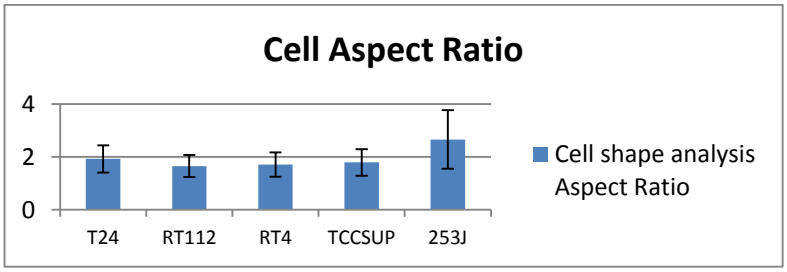


D

P-value, for parameters compared to the morphology of RT4 cells			
Cells	Area	Circularity	Aspect Ratio
T24	<0.001	<0.001	0.122403
RT112	<0.001	0.346577	0.606717
TCCSUP	<0.001	<0.001	0.550478
253J	<0.001	<0.001	<0.001

Figure 3-3 : **Quantitative analysis of the cell shape of bladder cancer cells in 2D culture on glass coverslips. The shape was manually outlined based on the F-actin staining: A)** Cell area. **B)** Cell circularity **C)** Cell aspect ratio. D) Student t-test was performed comparing the individual cells to the cell shape parameters of RT4 cells (Analysis performed on 30 cells, from 3 separate experiments).

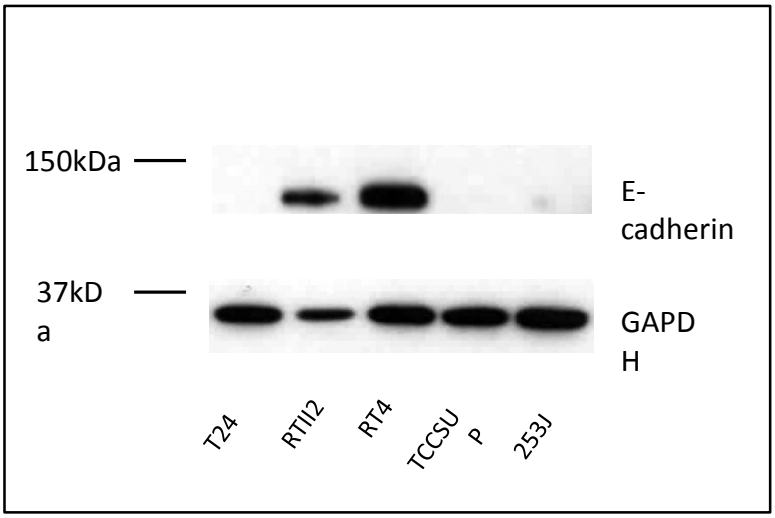
3.2.2 **Cadherins and cell: cell adherens junctions in bladder cancer cell lines**

Epithelial mesenchymal transition (EMT) is a process in which epithelial cells lose their characteristic polarity, disassemble cell-cell junctions and become more migratory (Radisky 2005, Baum, Settleman et al. 2008). Cadherin isoform switching (cadherin switching) involving loss of E-cadherin and gain of N-cadherin expression is characteristic in EMT, and tumour cells recapitulate this activity, resulting in aggressive tumour behaviour with the ability to invade and metastasize (Cavallaro 2004, Cavallaro and Christofori 2004, Wheelock, Shintani et al. 2008, Berx and van Roy 2009).

There is accumulating evidence that EMT and cadherin switching play important roles in bladder cancer invasion and metastasis. (Bringuier, Umbas et al. 1993, Bryan, Atherfold et al. 2008, Bryan and Tselepis 2010, Jager, Becker et al. 2010). It is speculated that Cadherin switching occurs late in the pathogenesis of bladder cancer, and contribute significantly to the divergent pathways of urothelial tumorigenesis.

Previous results (figures 3.1 - 3.3), suggest that T24, TCCSUP and 253J have undergone EMT, whereas RT4 and RT112 still retain their epithelial phenotype and markers. To support this hypothesis, the whole cell lysates of all 5 bladder cancer cell lines were screened for protein expression of E-cadherin and N-cadherin (figure 3.4). Consistent with the epithelial cell morphology seen previously, RT4 and RT112 cells retained the protein expression of E-cadherin, with no detectable expression of N –cadherin under basal growth conditions. Conversely, T24, TCCSUP and 253J cells have lost the protein expression of E-cadherin, and consistent with the archetypal cadherin switch model, have gained the expression of N-cadherin.

A



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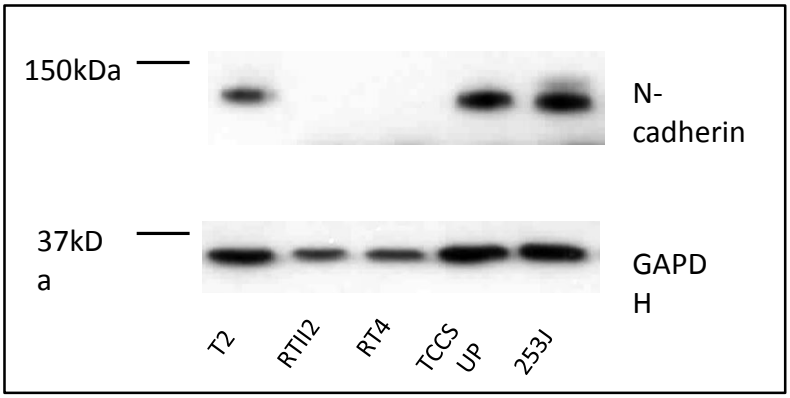


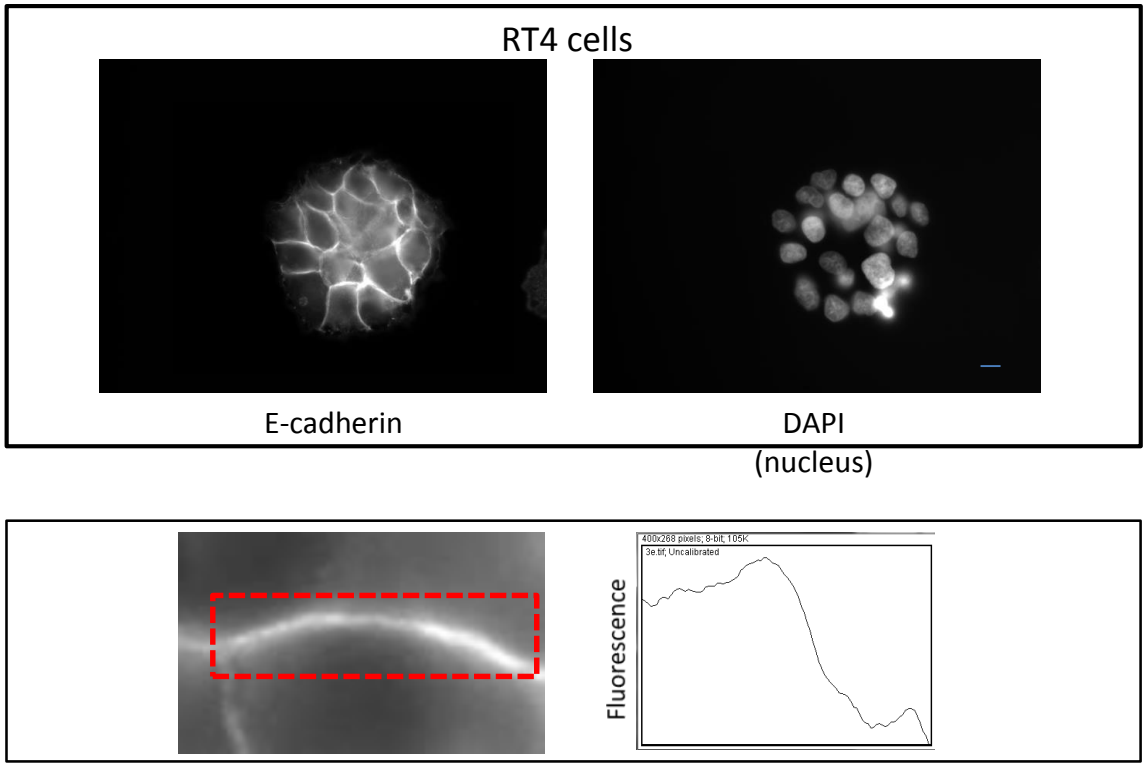
Figure 3-4 : **Cadherin switch in bladder cancer cell lines**. Whole cell lysates of bladder cancer cell lines were probed classic markers of cadherin switching in EMT. A) E-cadherin total protein level with GAPDH loading control. B) N-cadherin total protein level with GAPDH loading control. Images are representative of 3 independent experiments.

The expression and localisation of E-cadherin in RT112 and RT4 were also assessed by indirect immunofluorescence at basal growth conditions. In RT4 cells, the e-cadherin staining at cell-cell junction was uniform and evenly distributed to form distinct E-cadherin plaques along the areas of cell-cell contact margin (figure 3.5A). The e-cadherin staining in RT112 however displayed a more disrupted or 'zippered' appearance, where multiple E-cadherin-containing punctae cluster at varying intensities along the contact margins (figure 3.5B).

For cells forming epithelial sheets, three stages of cell-cell adhesion and colony formation have been proposed (Adams, Chen et al. 1998, Vasioukhin, Bauer et al. 2000, Vasioukhin and Fuchs 2001). Stage 1 corresponded with immature adhesions which loosely hold cells together. At stage 2, E-cadherin plaques develop at the edges of the contact, and at stage 3, the E-cadherin plaques cinch together to form multi-cellular vertices, further condensing the cell colonies, which indicated mature and stable cell-cell adherens junctions. I have used this analysis technique to estimate the maturity and stability of the epithelial junctions in RT4 and RT112 cells under basal growth conditions.

The fluorescence of E-cadherin signal was plotted to represent immunofluorescence intensity at the region of cell-cell contact in both these cell lines (figure 3.5). The formation of continuous of E-cadherin plaques at the margins of cell-cell contact in RT4 cells were consistent with more established and mature cell-cell junctions (stages 2-3), whereas the interrupted staining pattern in RT112 cells suggested less mature formation of epithelial cell-cell junctions(stages 1-2).

A



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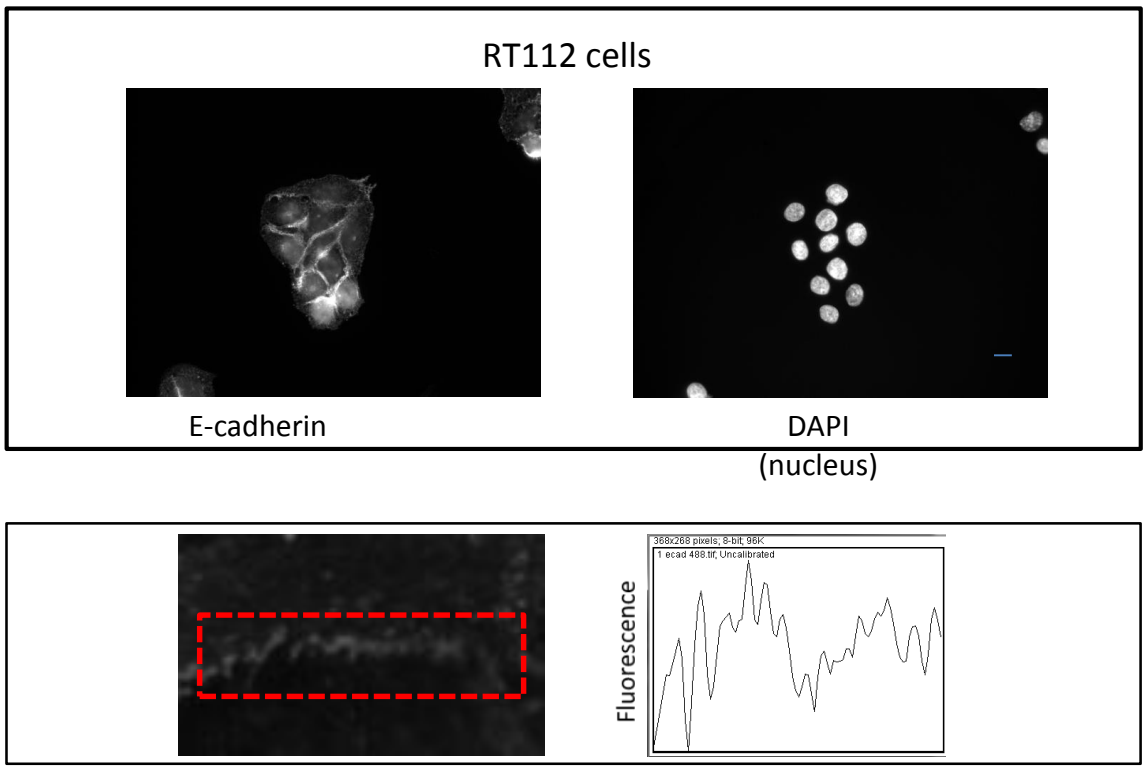


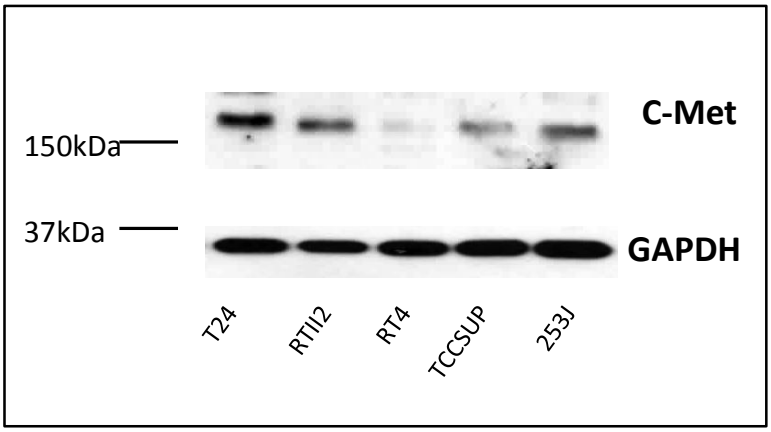
Figure 3-5 : **Characteristic of E-cadherin positive cell-cell junction of RT4 and RT112 cells in 2D culture:** Immunofluorescence and line scan of E-cadherin staining at cell: cell junction of RT4 (A) and RT112 (B) cells. The dashed red boxes approximately register the edges of the contact plotted for fluorescence (arbitrary unit). Images shown are representative of at least 3 independent experiments. Bar = 10μm

3.2.3 Changes in epithelial adherens junctions in RT112 and RT4 cells in response to HGF stimulation

Studies have shown that activation of tyrosine kinase receptor c-Met by its ligand, hepatocyte growth factor (HGF) in epithelial cells *in vitro* can induce changes in cell morphology, loss of cell polarity and dissociation of cell-cell junctions, associated with EMT (Stoker, Gherardi et al. 1987, Balkovetz, Pollack et al. 1997). There is also substantial body of evidence correlating HGF/C-Met signalling pathway to metastatic spread of tumours, including bladder cancer (Cheng, Trink et al. 2002) (Joseph, Weiss et al. 1995, Tamatani, Hattori et al. 1999, Wang, Nishitani et al. 2007). A member of group-2 PAK family, PAK4 was shown to act downstream of HGF, and PAK4 was required for HGF-induced scattering of prostate cancer cells (Wells, Abo et al. 2002, Ahmed, Shea et al. 2008). I therefore investigated whether these EMT-associated morphological changes in response to HGF could be reproduced in bladder cancer cells.

Although the expression of tyrosine kinase receptor c-Met has been studied in bladder cancer, the data available for all the cell lines as one panel were only available for the mRNA transcription, but not the protein expression (Cheng, Trink et al. 2002, Wang, Nishitani et al. 2007). I therefore proceeded to assess the expression level of c-Met in my panel of bladder cancer cell lines by Western blots (figure 3.6A). Within this panel, high protein levels of c-Met receptor were detected in T24 and 253J cells. RT4 cells expressed low level of c-Met. Moderate total protein levels of c-Met was observed in TCCSUP and RT112, and the highest level was seen in T24 (figure 3.6B).

A



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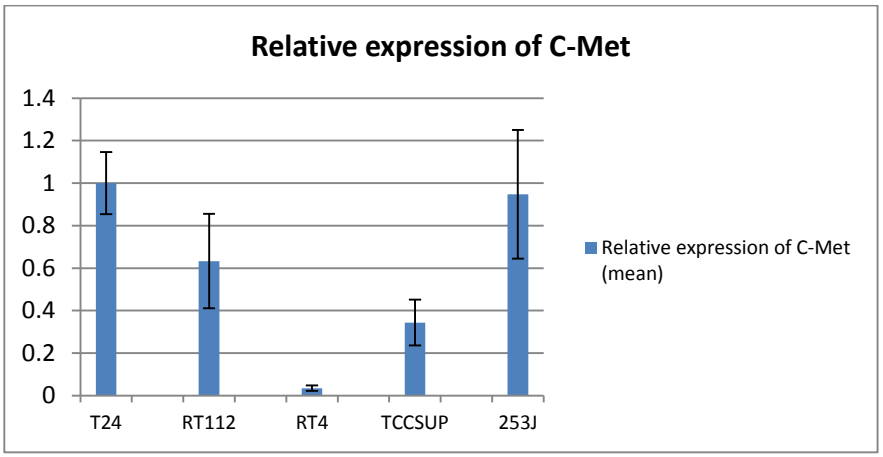

Figure 3-6 : **Protein expression of C-Met receptor in bladder cancer cell lines A)** Western blot of whole cell lysates of bladder cancer cell lines for C-Met Receptor, probed with Met (C-12) SCBT™ antibody. **B)** Analyses of C-Met Receptor expression on Western blot using densitometry quantification on ImageJ from 3 independent experiments

Having established that c-Met protein expression can be detected in bladder cancer cell lines, albeit at varying levels, I proceeded to investigate the response of 'epithelial-like' bladder cancer cell lines (RT112 and RT4) to HGF stimulation, and whether this assay can induce EMT-associated morphological changes. The cells were seeded on glass coverslips in full growth conditions at low density to allow formation of distinct cell colonies. The cells were then subjected to starve conditions for 24 prior to HGF stimulation. The cells were then stimulated by addition of HGF to the starve media, and fixed at different time-points following addition of HGF.

When the RT112 cells were stimulated with HGF using standard assay protocol, disruption of cell junctions was observed at 90 minutes, where the cell-cell junctions were disrupted. The cells displayed multiple slender membrane protrusions, within the newly created space as the cells dissociated from their epithelial sheet or colonies (figure 3.7).

The morphological changes observed in RT4 cells were not as pronounced as those manifested in RT112 cells. Following HGF stimulation, the cell contact within the RT4 epithelial sheet was maintained. However, the linear, homogenous E-cadherin plaques at the margin of cell-cell contact appeared more disrupted or 'zippered'. I also observed that HGF stimulation of RT4 cells resulted in formation of E-Cadherin punctate which aggregated along the E-cadherin plaque at the cell-cell adherens junctions, as well as 'granular' staining of E-cadherin in the cytoplasm (figure 3.8).

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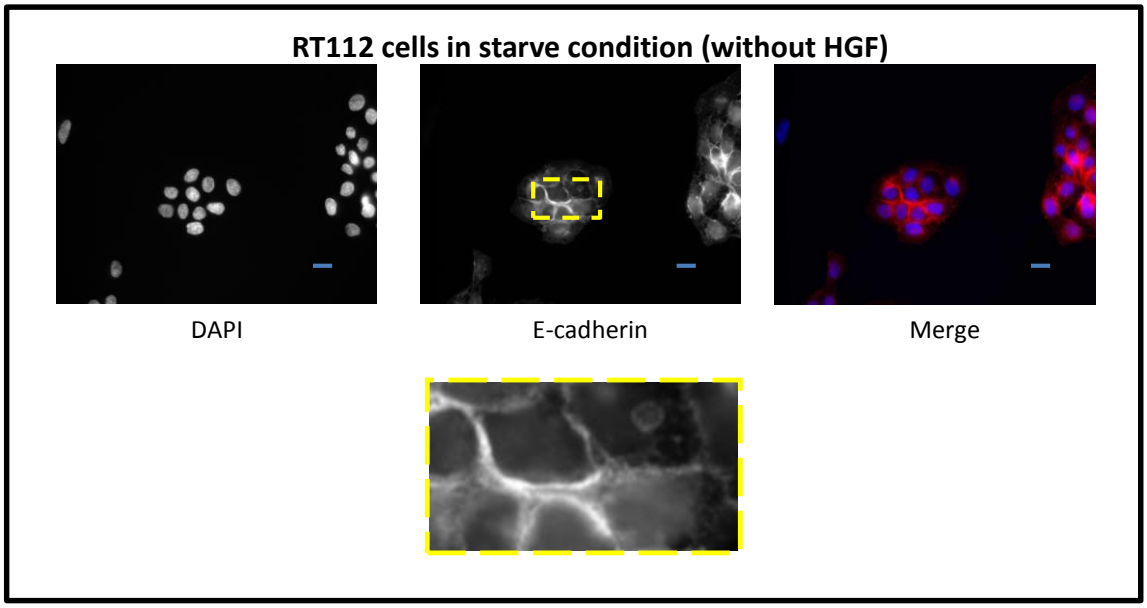

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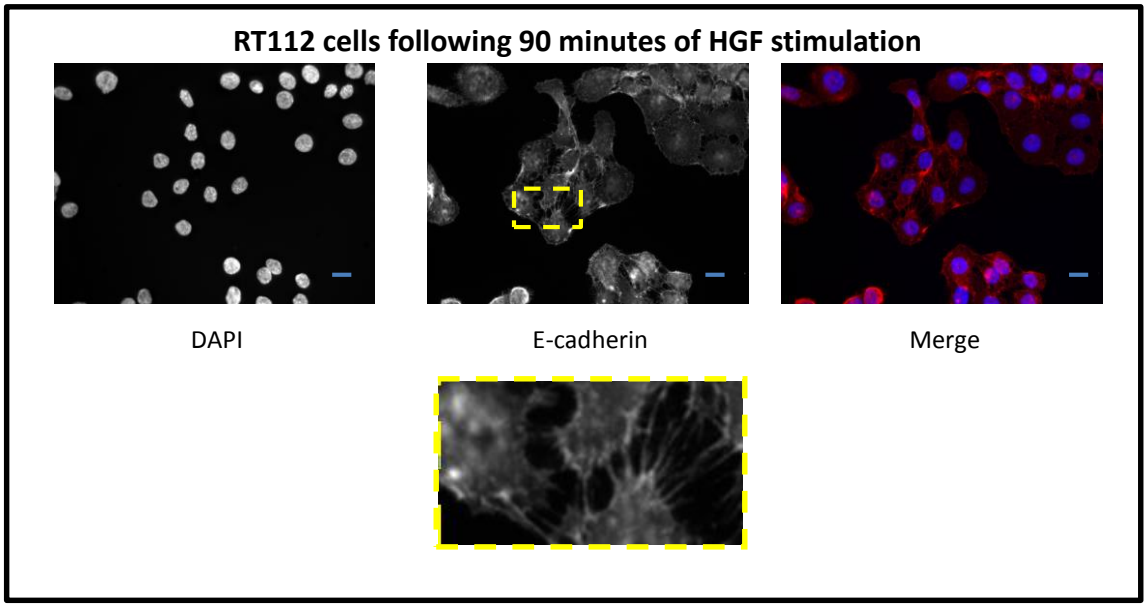

Figure 3-7 : **Disruption of cell adhesion following HGF stimulation in RT112 cells A)** E-cadherin staining in RT112 distribution of E-cadherin along the contact margins in RT112 cells. **B)** Following HGF stimulation, the cells dissociated from each other, and areas of cell: cell contact were replaced by multiple thin spikes of membrane protrusions. Magnified images of E-cadherin distribution at cell contact margins are boxed in yellow. Images shown are representative of 3 independent experiments. Bar = 10μm

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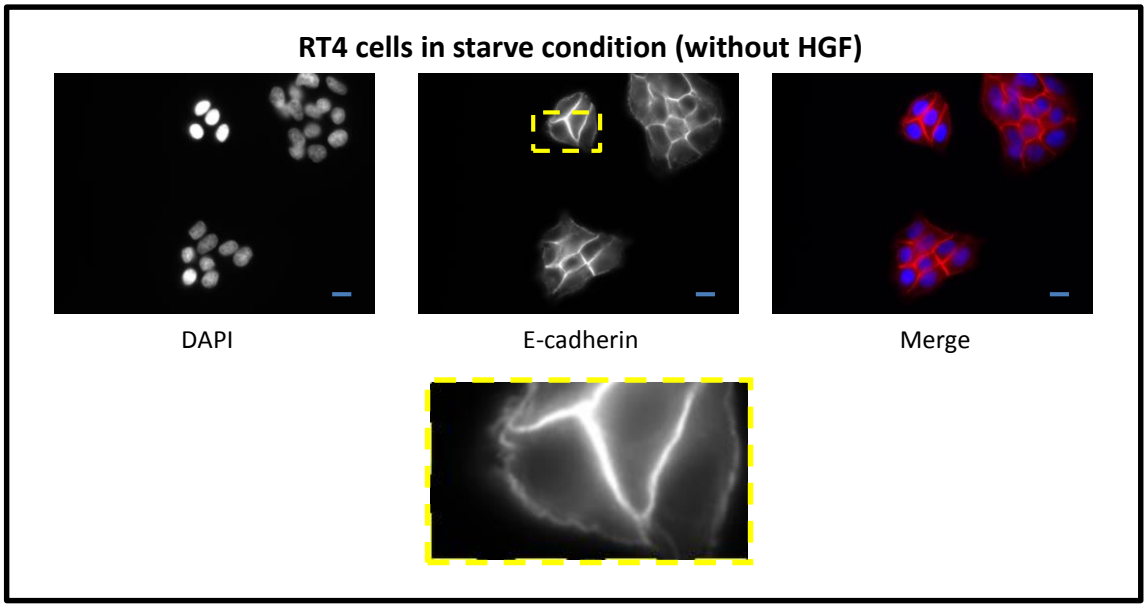

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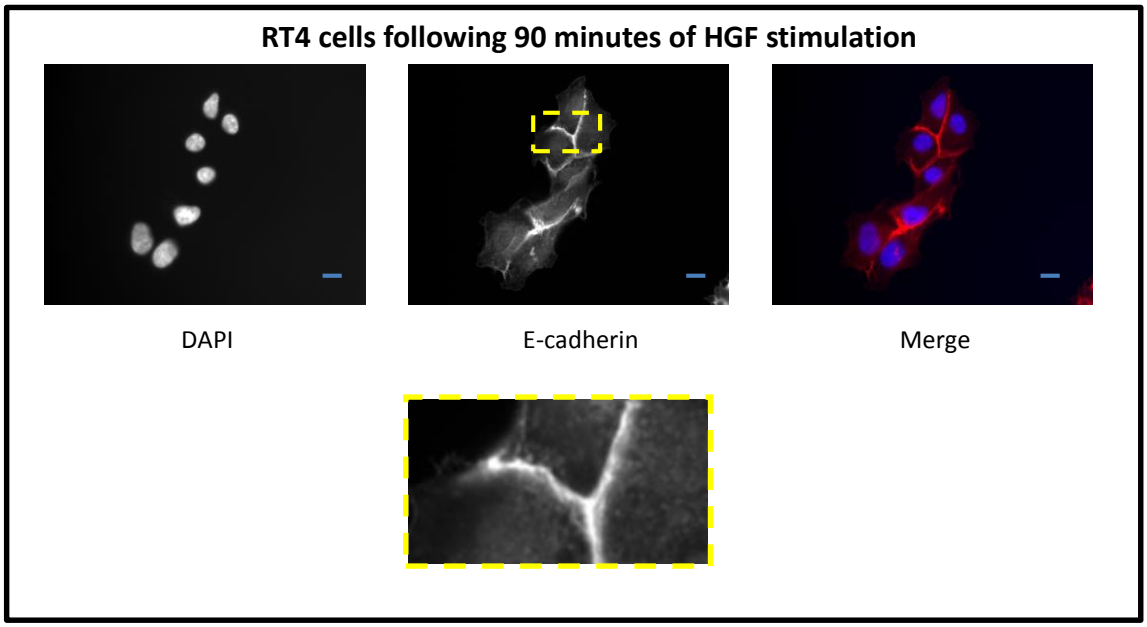

Figure 3-8 : **Changes in E-cadherin cell adherens junction following HGF stimulation in RT4 cells A)** Distinct cadherin staining appear as continuous plaques at margins of cell: cell contact in RT4 cells. **B)** Following HGF stimulation, subtle changes at the adherens junctions were characterised by appearance of E-cadherin punctae along the cell contact margin. Magnified images of E-cadherin distribution at adherens junction are boxed in yellow. Images shown are representative of 3 independent experiments. Bar = 10μm

3.2.4 **PAK1 expression in human bladder cancer cell lines**

PAK1 is the most comprehensively characterised member of the PAK-family. In a bladder cancer clinical study (Ito, Nishiyama et al. 2007), high expression of PAK1 at both gene transcription and protein expression levels were associated with high tumour grade, and increased risk of recurrence. Another clinical study, which analysed surgical specimens of patients with urothelial tumour of the upper urinary tract, had also demonstrated that over expression of PAK1, and activation of its upstream regulator, RAC1, were associated with increased risk of lymphovascular invasion and lymph node metastasis (Kamai, Shirataki et al. 2010).

One of the studies (Ito, Nishiyama et al. 2007) had also included functional analysis of PAK1 in bladder cancer cell lines 253J and EJ (previously reported to be identical to T24, (O'Toole, Povey et al. 1983)). The endogenous expression level PAK1 was reported to be relatively low in 253J compared to EJ cell line. Overexpression of constitutively active PAK1 (T423E) in 253J enhanced cell migration in wound healing and Matrigel migration assays. Conversely, suppression of endogenous PAK1 function in EJ cells by overexpression of kinase-dead PAK1 K299R delayed cell migration.

As PAK1 had been characterised in urothelial carcinomas, I have included the screen for the protein expression of PAK1 my panel of 5 bladder cancer cell lines. Western blots using PAK1 isoform-specific (Cell Signalling Technology ™) was performed , and I observed high total protein levels of PAK1 in RT4 and TCCSUP, moderate levels in 253J and T24, and very low protein expression of PAK1 in RT112 (figure 3.9). Even though PAK1 was differentially expressed, the protein levels of PAK1 *per se* did not show any direct correlation with the epithelial differentiation or invasive potential of the cell lines within this panel.

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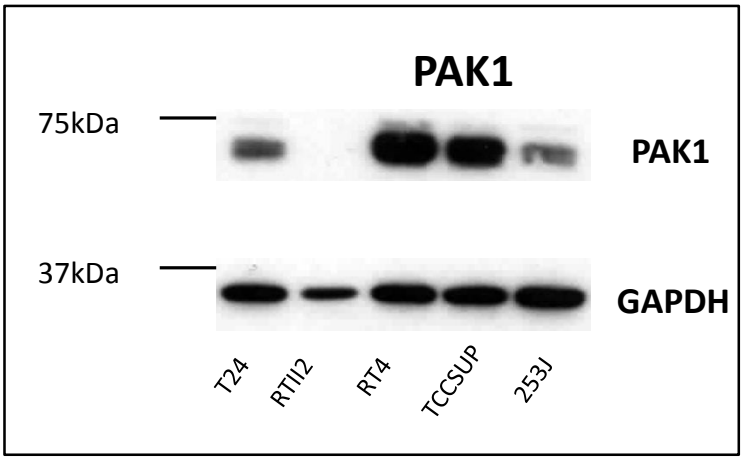

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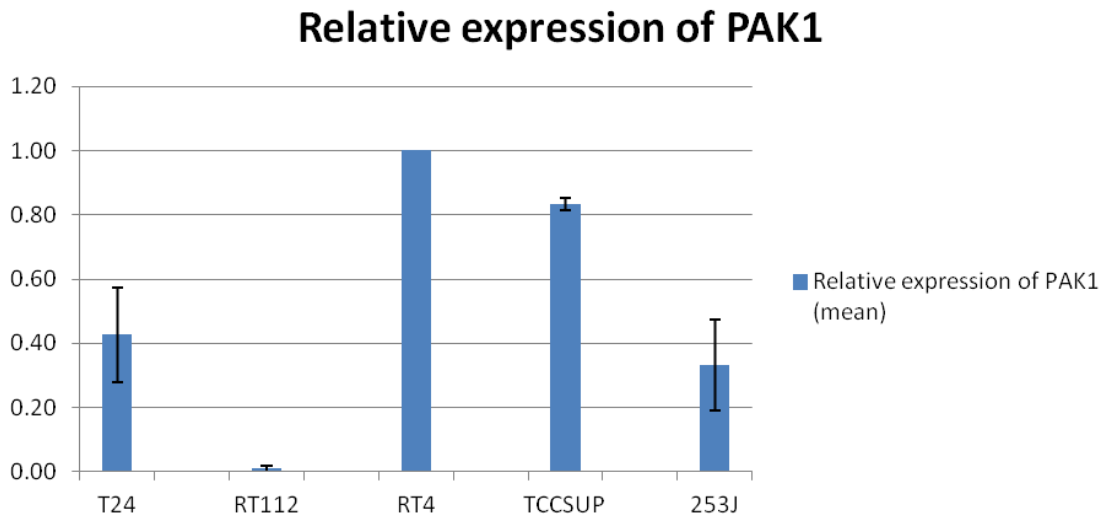

Figure 3-9 : **Protein expression of PAK1 in bladder cancer cell lines A)** Western blot of whole cell lysates of bladder cancer cell lines probed with PAK1 antibody. **B)** Analyses of PAK1 expression on Western blot using densitometry quantification on ImageJ from 3 independent experiments.

3.2.5 PAK4 expression in bladder cancer cell lines

Group-2 members of the PAK family are structurally different from group-1 PAKs (*figure of PAK structure in introduction chapter* 1). The evidence for the role of group-2 PAKs in oncogenic transformation and in cellular processes associated with cell survival, proliferation, cytoskeletal organisation have been described in a number of studies (Gnesutta, Qu et al. 2001, Gnesutta and Minden 2003, Eswaran, Soundararajan et al. 2009). PAK4 is the first identified member of group-2 PAKs (Abo, Qu et al. 1998). Among members of group 2 PAKs, PAK4 has been most closely linked to cancer, and has been shown to be upregulated or overexpressed in a number of human malignancies (Dart and Wells 2013, Whale, Dart et al. 2013, Wong, Chen et al. 2013). There is currently no research in publication with strong evidence associating PAK4 in the oncogenesis of bladder cancer.

I screened my panel of bladder cancer cell lines for PAK4 expression using in-house PAK4 specific polyclonal antibody which had been previously validated (Wells, Whale et al. 2010). The antibody detects PAK4 at MW 65 kDa. PAK4 was expressed in all bladder cancer cell lines on my panel. The expression profile of PAK4 across the cell lines were ubiquitous, and did not differentiate between the cell lines of highly invasive origin and low grade papillary origin (figure 3.10).

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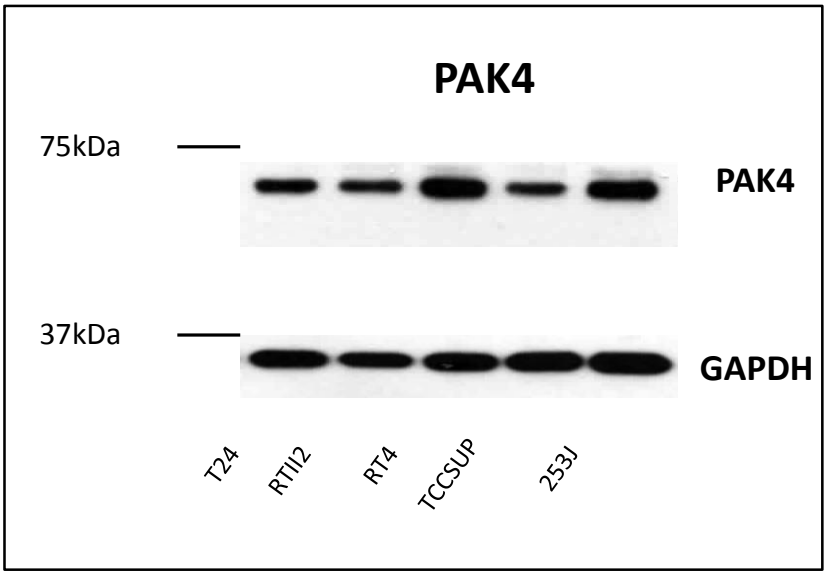

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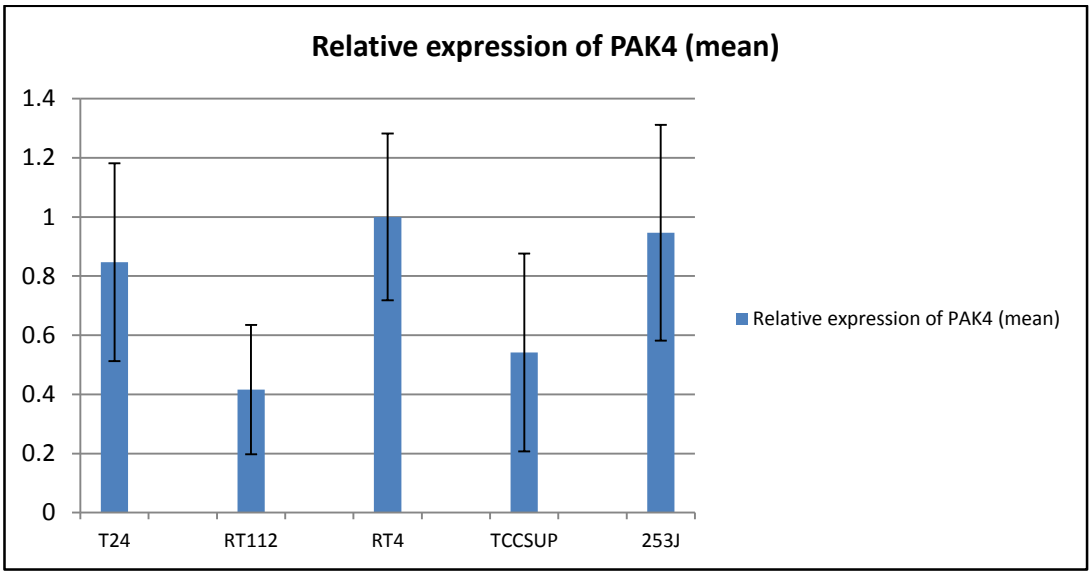


Figure 3-10 : **Protein expression of PAK4 in bladder cancer cell lines A)** Western blot of whole cell lysates of bladder cancer cell lines probed with PAK4 (in-house) antibody. **B)** Analyses of PAK4 expression on Western blot using densitometry quantification on ImageJ from 3 independent experiments.

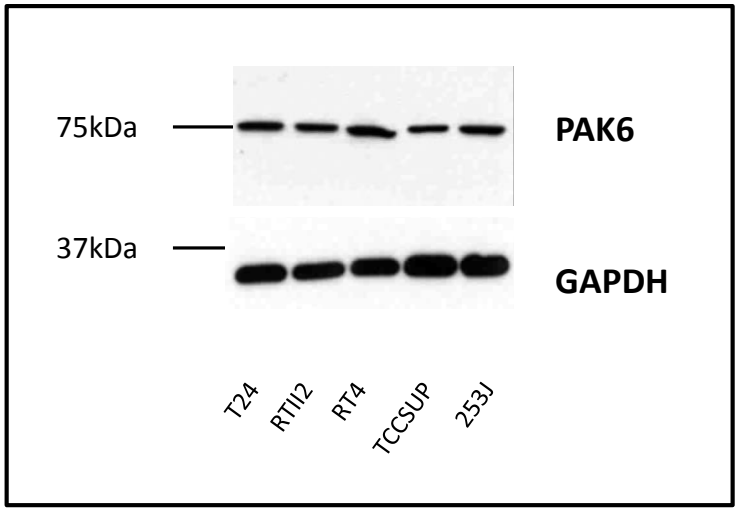
3.2.6 PAK6 expression in bladder cancer cell lines

PAK6 is also a member of the group 2 PAKs with suggested roles in cancer. PAK6 was first identified as an androgen receptor (AR) interacting protein (Yang, Li et al. 2001, Lee, Ramos et al. 2002). PAK6 overexpression had been shown in prostate and breast cancer cell lines, and increase in PAK6 protein expression has been associated with failure of androgen deprivation therapy in prostate cancer patients (Kaur, Yuan et al. 2008). Recently, PAK6 overexpression was shown to cause disruption of cell-cell junctions in androgen receptor (AR) negative DU145 prostate cancer cells downstream of HGF (Fram, King et al. 2013). However, *in vivo* study had also demonstrated that PAK6 may inhibit prostate tumour growth in mice by regulating AR homeostasis (Liu, Li et al. 2013).

The role for PAK6 in bladder cancer has not been described. However, the evidence for androgen receptor regulation in urothelial oncogenesis is emerging (Miyamoto, Yao et al. 2012, Hsu, Hsu et al. 2013), supported by the epidemiological data for gender-specific differences in the incidence and progression of bladder cancer.(Siegel, Naishadham et al. 2012). The role of PAK6 in AR regulation had raised the question whether PAK6 may also play a role in bladder cancer epithelial mesenchymal transition.

I proceeded to screen for PAK6 expression in my panel of bladder cancer cell lines. Whole cell lysates of bladder cancer cell line cultured in basal growth condition were assayed to detect the expression level of PAK6 using isoform specific antibody obtained from Gene Tex (GTX30295). This antibody detected PAK6 at 75kDa, and did not display any additional bands of protein which may suggest cross reaction with other members of the PAK family. Ubiquitous PAK6 protein expression was observed in all bladder cancer cell lines (figure 3.11A). ImageJ densitometry quantification of Western blots from 3 separate sets of cell lysates indicated that PAK6 expression was homogenous in this panel of bladder cancer cell lines (figure 3.11B), and did not show differential expression between the epithelial and mesenchymal bladder cancer cell lines.

A



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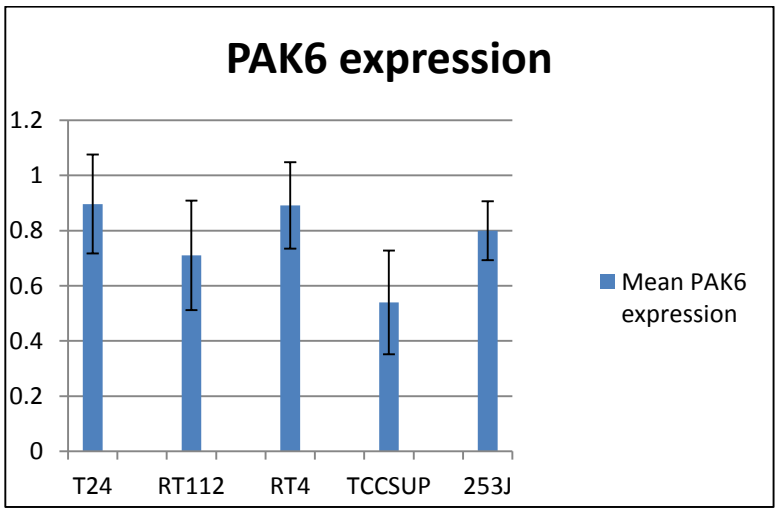

Figure 3-11 : **Protein expression of PAK6 in bladder cancer cell lines A)** Western blot of whole cell lysates of bladder cancer cell lines probed with PAK6 isoform specific antibody. **B)** Analyses of PAK6 expression on Western blot using densitometry quantification on ImageJ from 3 independent experiments.

3.2.7 **Generation of PAK5 isoform specific rabbit polyclonal antibody**

PAK5 is a novel member of the PAK family, and differentially in normal human tissue, with the highest expression levels in the brain (Dan, Nath et al. 2002, Pandey, Dan et al. 2002). A number of studies had linked PAK5 with tumour development, invasion and metastasis. PAK5 overexpression had been described in colorectal cancer (histopathology staining), and associated with invasion and metastasis (Gong, An et al. 2009). Gain of function (GOF) mutation of PAK5 in lung cancer cell line H2097 enhanced the activation of ERK pathway to maintain cell proliferation and viability (Fawdar, Trotter et al. 2013). PAK5 has also been studied in the context of pancreatic cancer, gastric cancer, hepatocellular carcinoma, ovarian cancer and glioma, and associated with tumour proliferation and/or invasion (Giroux, Iovanna et al. 2009, Gu, Li et al. 2013, Han, Wang et al. 2013, Li, Yao et al. 2013, Wang, Cheng et al. 2013).

A number of commercial antibodies were initially tested to detect the protein expression of PAK5 in bladder cancer cell lines. The detection of PAK5 using commercially available antibodies was inconsistent and unreliable (data not shown). Even though PAK5 is a relatively novel protein and has not been extensively studied, there were already data on Human Protein Atlas (http://www.proteinatlas.org/ENSG00000101349/tissue) accessed in May 2011 which indicated that protein levels of PAK5 were detectable in immunostaining of some normal and malignant urothelial tissues. In order to probe for PAK5 expression in bladder cancer cell lines, I generated a PAK5 isoform-specific rabbit polyclonal antibody (referred to as in-house PAK5 antibody).

Protscale software available on Expasy website http://web.expasy.org/protscale/ was used to identify the suitable peptide sequence on PAK5 for antigen recognition. Two different scales (Hopp & Woods, and Kyte & Doolittle) were used to assess the hydrophobicity and the hydrophilicity profiles of PAK5, which helped to identify the most suitable epitope for antigen presentation (figure 3.12A).

A synthetic peptide sequence (YREKS LYGDD LDPYY) corresponding to aa146-160 of PAK5 protein, located in the N-terminal regulatory domain was selected. The Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/) was used to check for other proteins which contain similar peptide sequence, and may also interact with the antibody designed. The search using Protein BLAST showed that this peptide sequence was specific for PAK5. The location of the epitope I had selected for PAK5 antibody production was similar, but not identical to the epitope for PAK5 antibody previously designed by another group, which corresponded to amino acids 139-157 of human PAK5 (Wu and Frost 2006).

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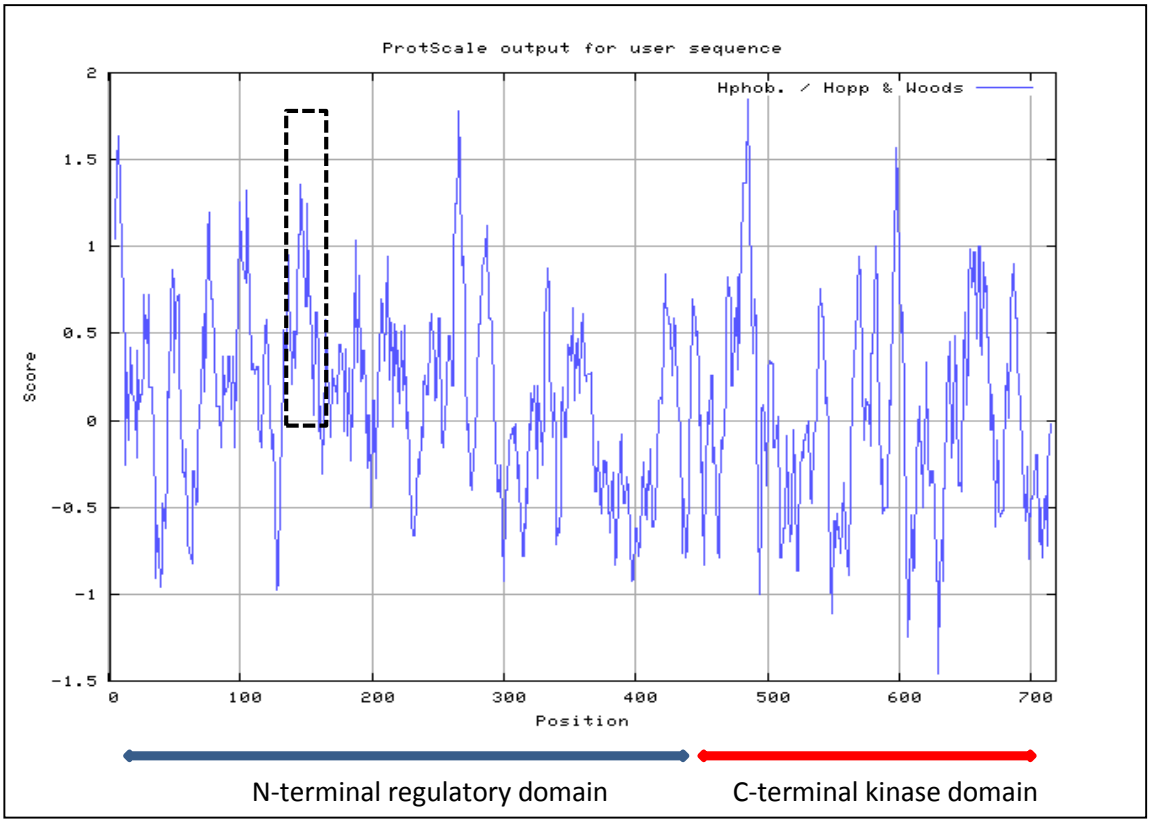

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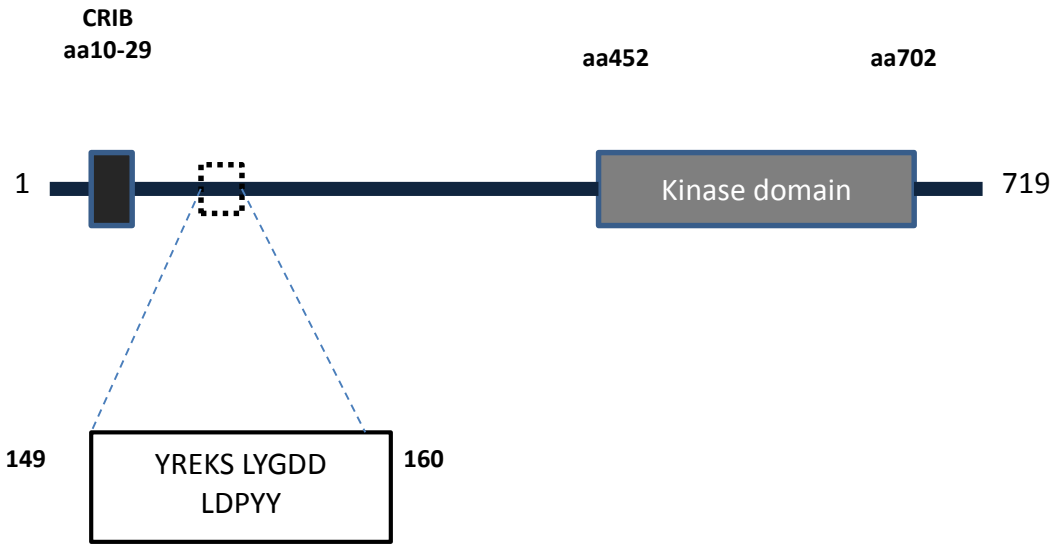

Figure 3-12 **: Generation of PAK5 in-house antibody A)**Hopp and Woods hydrophobicity /hydrophilicity profile of PAK5 protein used for epitope selection (boxed).B) Epitope for antibody presentation (boxed) within the N-terminal regulatory domain of PAK5

3.2.8 Validation of in-house PAK5 antibody and generation of PAK5-fusion proteins

In order to validate the isoform-specific PAK5 antibody, I generated recombinant PAK5 by sub cloning the DNA into GFP and Myc expression vectors (see appendix 4 and method section 2.2.3) using Gateway™ Cloning Technology. The recombinant GFP-PAK5 and Myc-PAK5 plasmid DNAs were sequenced. The results from DNA sequencing analysis confirmed that no mutations had occurred throughout the process, and the DNA matched exactly to the available sequence of PAK5 (also known as PAK7) on Human ORFeome Database (http://horfdb.dfci.harvard.edu).

I overexpressed the PAK5-fusion proteins in T24 cells to obtain the whole cell lysates. The whole cell lysates was first probed with one of the commercial antibodies (SCBT SC-16 PAK5 antibody) which had been validated for detection of overexpressed PAK5, documented on the manufacturer's product datasheet. Both Myc-PAK5 and GFP-PAK5 recombinant proteins were detected by this antibody (figure 3.13A). I proceeded to assess whether the in-house rabbit polyclonal PAK5 antibody can sensitively detect these recombinant proteins. Western blots confirmed that the antibody was able to detect the bands at approximately 81 kDa for Myc-PAK5 (figure 3.13B), and at 110 kDa for GFP-PAK5 (figure 3.13C)

I assessed the specificity of the antibody by testing the antibody with whole cell lysates of T24 cells over-expressing recombinant GFP-fusion PAK4 and PAK6 proteins, as PAK4 and PAK6 are structurally closely related to PAK5. The in-house PAK5 detected GFP-PAK5 but not GFP-PAK4 or GFP-PAK6 (figure 3.13B). I noted that there was no endogenous PAK5 signal detected in T24 (figure 3.13A-C). The same sets of whole cell lysates were also probed with GFP-specific antibody for to show the expression of all GFP-fusion (PAK4, PAK5 and PAK6) proteins (figure 3.13C). Taken together, the new PAK5 in-house antibody was sensitive to detect PAK5, and specific that it did not detect to either PAK4 or PAK6.

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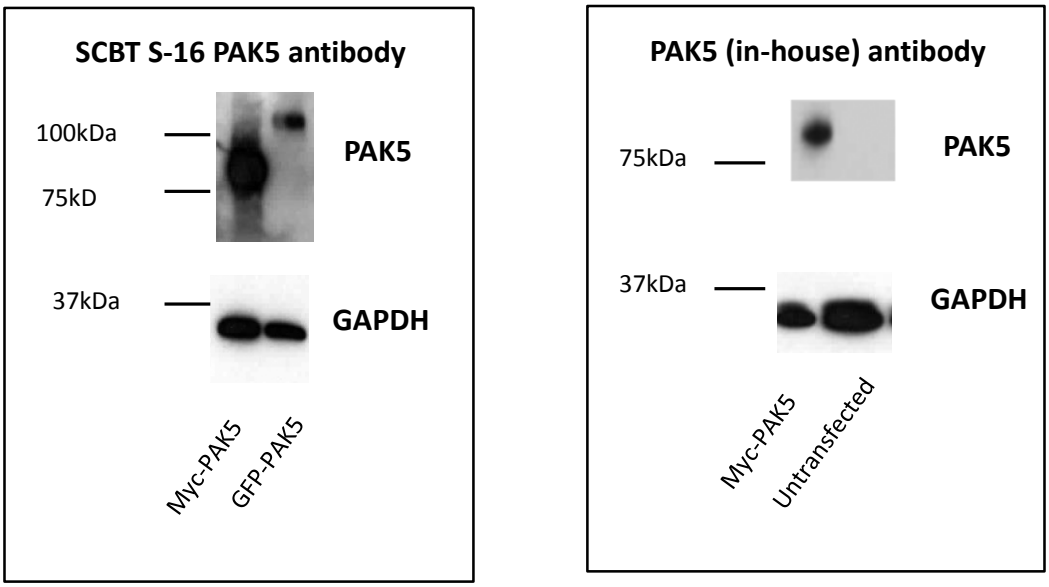

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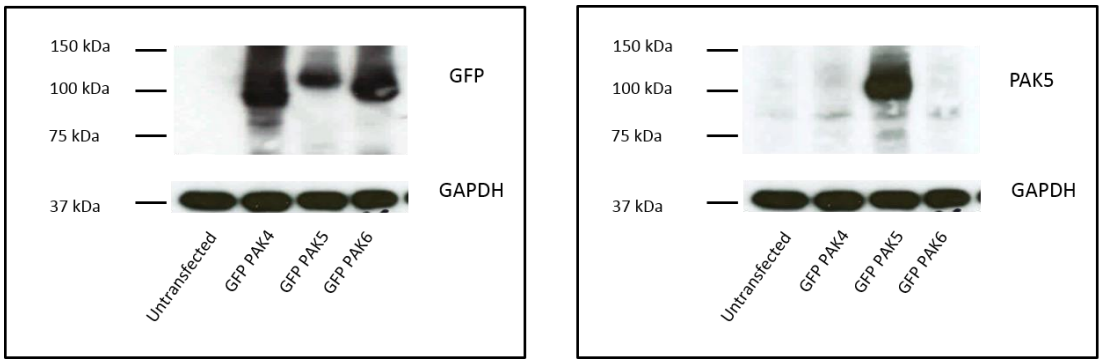

Figure 3-13 : **Generation of recombinant PAK5-fusion proteins and validation for PAK5 in-house antibody A)** Detection of GFP PAK5 and Myc-PAK5 at the estimated molecular weights. **B)** Detection of recombinant GFP-PAK5 protein overexpressed in T24 cells, but not GFP-PAK4 or GFP-PAK6 on Western blot using PAK5 in-house antibody. **B)** GFP antibody recognised all GFP-tagged recombinant Group-2 PAKs at the estimated MW (with the addition of 27kDa to account for GFP tag) in the same set of lysates as **B**

3.2.9 PAK5 protein expression is associated with epithelial differentiation of bladder cancer cell lines

Having established the sensitivity and specificity of the in-house PAK5 antibody, I proceeded to screen the bladder cancer cell lines for endogenous expression of PAK5 by Western blotting. The antibody detected endogenous PAK5 protein at the estimated molecular weight of 81kDa (figure 3.15).The endogenous PAK5 expression was strongest in RT4, followed by RT112 cells. Very low expression level of PAK5 was detected in T24, TCCSUP and 253J cells. The protein expression profile of PAK5 was reminiscent of the expression profile of E-cadherin, and inversely correlated to the N-cadherin expression, previously seen in for this panel of cell line (figures 3.5 A & B).

The differential expression of PAK5 in bladder cancer cell lines which correlated with Cadherin switching is a novel and interesting finding. This correlation (direct or inverse) had not been identified in the screen for PAK1, PAK4 or PAK6 in bladder cancer cell lines. Based on these results, I decided to focus my research on the role of PAK5 in the context of bladder cancer specifically, and loss of epithelial differentiation in general. The results of further experiments were presented, analysed and discussed in the next sections.

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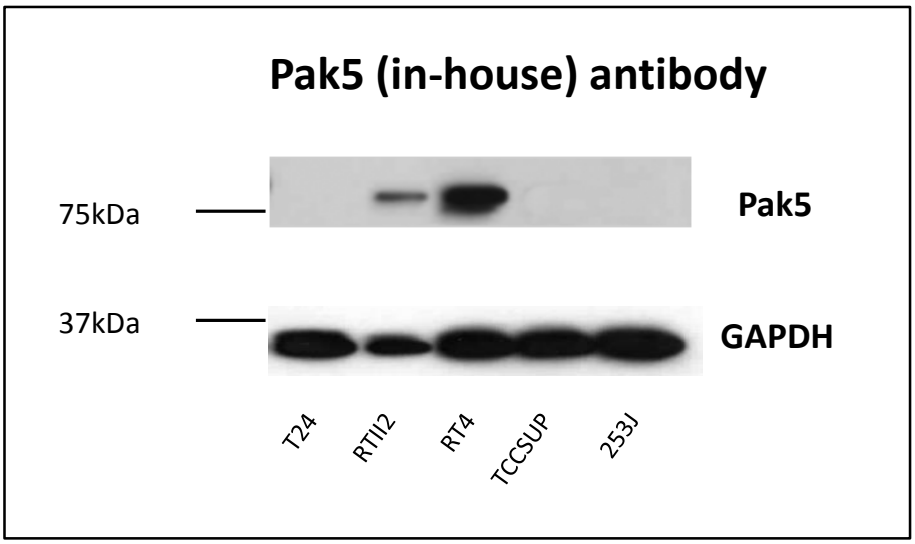

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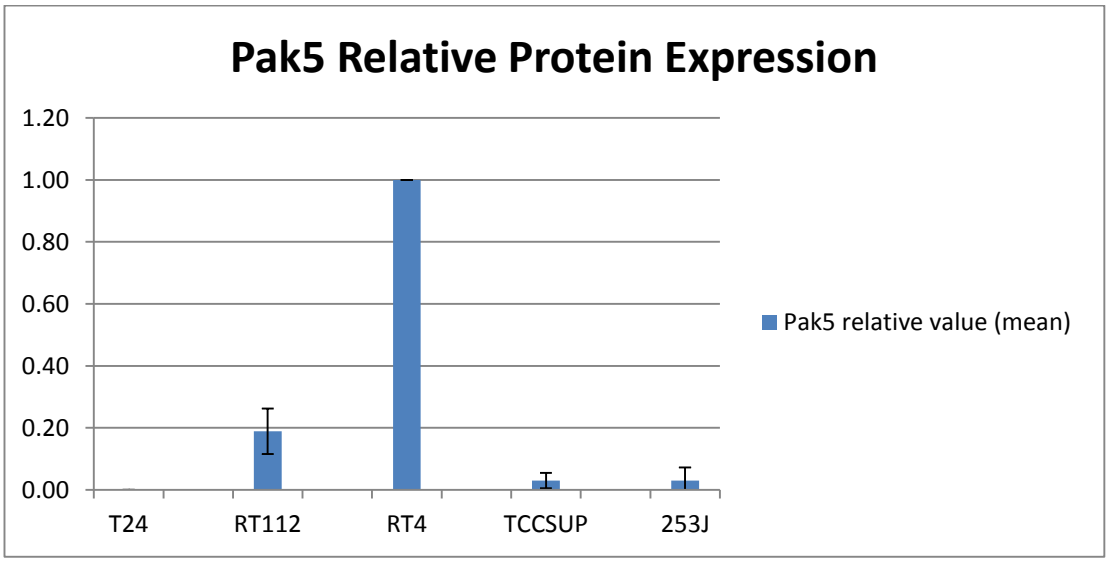


Figure 3-14 : **Protein expression of PAK5 in bladder cancer cell lines A)** Western blot of whole cell lysates of bladder cancer cell lines for PAK5, probed with PAK5 in-house antibody. **B)** Analyses of endogenous PAK5 expression on Western blot using densitometry quantification on ImageJ from 3 independent experiments.

3.2.10 Endogenous PAK5 expression in also correlates with E-cadherin expression in breast and pancreatic cancer cell lines

I previously observed that the protein expression of PAK5 positively correlated with the expression of E-Cadherin in bladder cancer cell lines. Therefore, in order to investigate whether the similar correlation occur in carcinoma cell lines from other organs, I screened for the expression of PAK5 and E-Cadherin in selected breast and pancreatic cancer cell lines with distinct epithelial or mesenchymal morphology.

Breast cancer cell line MDA-MB231 was selected to represent mesenchymal breast cancer cell line, with loss of E-Cadherin protein expression, whilst MCF7 cell line represented carcinoma cells with moderately differentiated epithelial morphology and protein expression of E-Cadherin (Lombaerts, van Wezel et al. 2006). Human mammary epithelial cell line, MCF10A was also included in this panel to represent cells with distinct epithelial phenotype (Maeda, Johnson et al. 2005).

Pancreatic cancer cell lines PaTu 8898T and PaTu 8898S were included in the panel due to their different morphological properties and EMT status, despite their common origin from the same metastatic tumour (liver metastasis) of pancreatic adenocarcinoma (Elsasser, Lehr et al. 1992). PaTu 8898T cells has mesenchymal growth characteristic while PaTu 8898S has distinct epithelial morphology and conserved cell-cell junctions at basal growth conditions (Belo, van der Sar et al. 2013).

Similar to bladder cancer cell lines panel, the positive association between PAK5 and E-Cadherin was also observed within this selection of cell lines of breast and pancreatic origins (figure 5.1). Higher protein expression level of PAK5 was detected in cells with conserved epithelial morphology and protein expression of E-Cadherin (RT4, MCF7, MCF10A and PaTu 8898s). The protein expression of PAK5 was undetectable in cells with mesenchymal morphology with loss of E-cadherin expression (T24, MDA MB231 and PaTu 8898T).

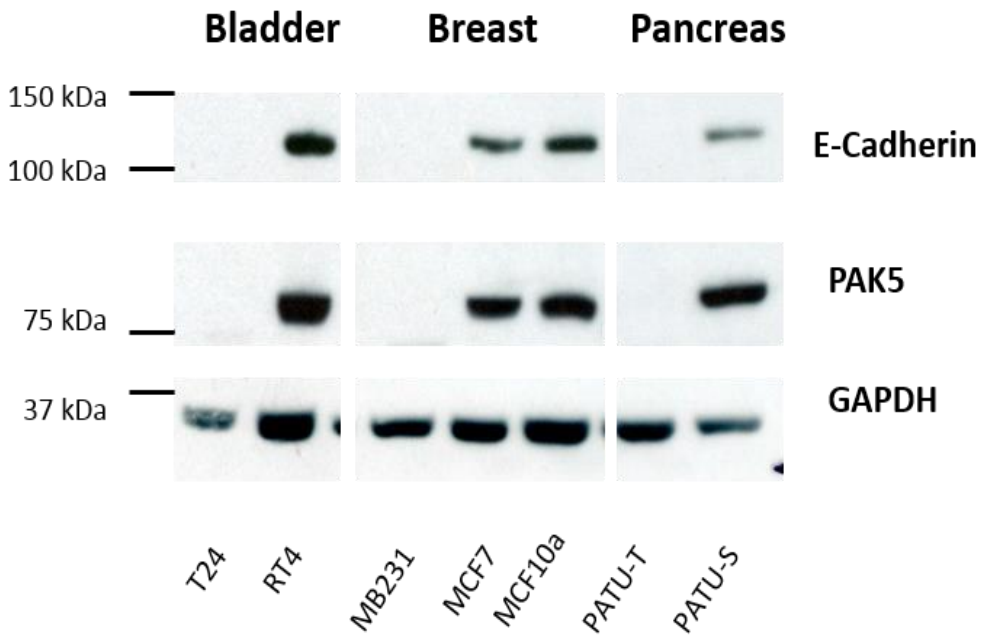


Figure 3-15 **: Expression of PAK5 and E-Cadherin in cancer cell lines with epithelial and mesenchymal morphology**. Whole cell lysates of breast and pancreatic tumours were probed for protein levels of E-Cadherin and PAK5 with GAPDH as loading control. Lysates of bladder tumour cell lines T24 and RT112 were included as positive and negative indicators. Western blots of relative protein expressions were representative of 3 independent experiments.

3.3 Discussion

The initial aim of this project was to identify a member of the group 2 PAK family that exhibit differential protein expression in bladder tumours that may correlate with invasive potential. I have used bladder cancer cell lines in this study. The advantages of using cell lines include the (almost) unlimited supply of cells derived from each human tumour, and the stability of many phenotypes in standardised culture conditions. The disadvantages include cross-contamination between the cell lines, spontaneous transformation, and that certain characteristics are not stable in long term culture conditions.

Characterisation of the epithelial morphology of bladder cancer cell lines

It is important that the bladder cancer cell lines used in this project be independently characterised to assure the validity and reproducibility of the experimental results presented in this theses. The bladder cancer cell lines in the screen were matched to represent different stages of urothelial tumour progression based on their epithelial or mesenchymal phenotypes. The state of malignant transformation was characterised by looking at the arrangement of actin cytoskeleton, cell shape and formation (or disruption) of cadherin-based cell: cell adherens junctions.

I have compared my findings on the descriptive cell morphology and the Cadherins expression with a study which specifically addressed the subject of EMT in the context of bladder cancer cell lines (Baumgart, Cohen et al. 2007). The study by Baumgart el al included RT4, RT112, TCCSUP and EJ (identical to T24 (O'Toole, Povey et al. 1983)) bladder cancer cell lines, and the descriptive classification of epithelial or mesenchymal morphology, and the expression of EMT markers (E-cadherin and N-cadherin) were consistent to the phenotypes displayed by my panel of cell lines. Although the definition and occurrence of EMT *in vivo* remain controversial (Tarin, Thompson et al. 2005), the conceptual framework embracing the loss of epithelial markers and gain of mesenchymal markers have been associated with bladder cancer progression in a number of studies (Mialhe, Levacher et al. 2000, Baumgart, Cohen et al. 2007, Bryan, Atherfold et al. 2008).

In section 3.2.3, I assessed the response of RT112 and RT4 cells to (paracrine) HGF-stimulation as a functional assay to induce cell-cell junction dissociation which may mimic the process of EMT. The dissociation of E-cadherin based cell-cell junctions in RT112 was more pronounced that the changes observed in RT4 cells colonies. RT112 cells, with less mature cell-cell junctions, may be considered to be in the intermediate state of EMT. Intermediate-stage cells harbour

greater plasticity to progress to a mesenchymal phenotype (Huang, Guilford et al. 2012), as observed in these 2 cell lines following HGF stimulation assay. Assessing the possible utility for HGF stimulation assay was very relevant to this study, as 2 members of the group 2 PAK family, PAK4 and PAK6, have been demonstrated to function downstream of HGF to induce cell-cell dissociation in prostate cancer cell line with epithelial morphology (Wells, Abo et al. 2002, Wells, Whale et al. 2010, Fram, King et al. 2013).

My independent assessment of the actin characteristics, Cadherin isoforms expression profile, and the differential response to HGF stimulation suggest that in this panel of bladder cancer cell lines, RT4 cells represent well differentiated urothelial tumours and RT112 cells represent moderately differentiated tumours. The expression of N-cadherin (cadherin switch) and mesenchymal morphology in T24, 253J and TCCSUP would suggest invasive and metastatic potential of these cell lines. Taken together, despite more than 3 decades of culture, storage and propagation, these cell lines still represented the primary tumours from which they were originally cultured.

Protein expression of PAK isoforms in bladder cancer cell lines

Having characterised the bladder cancer cell lines to correspond with different stages of EMT and their invasive potential, I proceeded to screen for the protein expression of PAK1, and all 3 isoforms of group 2 PAKs. The screen for PAK1 protein expression in bladder cancer cell lines was included as an arbitrary positive control, as PAK1 is the only member of the PAK-family that has been associated with urothelial tumorigenesis.

PAK1 has been studied in urothelial carcinoma of the bladder and the upper urinary tract, and its overexpression was associated with poor clinical prognosis. In a bladder cancer clinical study (Ito, Nishiyama et al. 2007), high expression of PAK1 at both gene transcription and protein expression levels were associated with high tumour grade, and increased risk of recurrence. Another clinical study which analysed surgical specimens of patients with urothelial tumour of the upper urinary tract, had also demonstrated that protein over-expression of PAK1, assayed by Western blotting and immunohistochemistry, was associated with increased risk of lymphovascular invasion and lymph node metastasis (Kamai, Shirataki et al. 2010).

One of the studies (Ito, Nishiyama et al. 2007) had also included information for PAK1 in bladder cancer cell lines 253J and EJ. EJ cell line had previously reported to be identical to T24 (O'Toole,

Povey et al. 1983). The endogenous expression level PAK1 (Western blot, antibody SCBT sc-881) was reported to be relatively low in 253J compared to EJ (T24) cell line. I did not find significant difference in PAK1 protein expression assay on Western blotting (figure 3.10) between 253J and T24 did not significantly differ as previously reported. This discrepancy may be due to the cell stock used, culture conditions, or the difference in the isoform specific PAK1 antibody used.

Even though differential expression profile was noted within my panel of 5 cell lines, it did not correlate with the EMT characteristic of the cells. Despite the lowest expression level in the epithelial RT112 cells, the highest expression level of PAK1 was also detected in RT4, a cell line with well differentiated epithelial morphology. This finding was counterintuitive. Based on the previous publications on PAK1 in bladder cancer, I would have predicted that PAK1 expression would be the lowest in RT4 and RT112, and higher in the mesenchymal T24, 253J and TCCSUP.

It could be speculated that the expression of total protein for PAK1 may not directly correlate with the activity of PAK1. PAK1 exists as inactive autoinhibited homodimers, where the two kinase domains from 2 different PAK1 molecules inhibit one another (Lei, Lu et al. 2000, Pirruccello, Sondermann et al. 2006). Upstream regulation of PAKs involves GTP-Cdc42 or Rac binding to the regulatory domain, and its displacement, which allows autophosphorylation of the kinase domain and maximal activation. Other members of group-1 PAKs, PAK2 and PAK3 are structurally very similar to PAK1, and regulated by GTP-dependent upstream molecular switches in a similar manner to PAK1 (Molli, Li et al. 2009). In contrast to group 1 PAKs, the interaction of group 2 PAKs with Rho GTPases may not affect the kinase activity; instead, the interaction localises PAKs to specific intracellular structures, possibly bringing the PAKs into proximity with the substrates (Cotteret, Jaffer et al. 2003, Wu and Frost 2006). A number of studies had also demonstrated that overexpression of wild type group 2 PAKs could activate common pathways downstream of PAKs such as LIMK-Cofilin (Dan, Kelly et al. 2001) and JNK pathways (Dan, Nath et al. 2002), more efficiently than wild type group 1 PAKs, where overexpression of the constitutively active forms of group 1 PAKs, or co-expression of GTP-bound Rho GTPases were usually required. The observations indicate that group 2 PAKs may be constitutively active in basal condition, and regulated differently to group 1 PAKs.

Despite the poor correlation of PAK1 protein expression (intended arbitrary positive control) and the EMT stages of bladder cancer cell lines, I proceeded to screen for the expression profile of group 2 PAKs. Two members of group-2 PAK proteins, PAK4 and PAK6, were ubiquitously expressed in all 5 bladder cancer cell lines. The expression profile for PAK4 and PAK6 did not show any distinct differentiation or correlation between mesenchymal and epithelial cancer cell lines, which implied that the expression of these PAKs did not directly activate invasion and metastasis in bladder cancer. The role for PAK4 or PAK6 in the urothelial oncogenesis however could not be excluded as they may contribute to other cancer hallmark capabilities such as resisting cell death or sustaining proliferative signalling (Radu, Semenova et al. 2014).

The protein expression profile of PAK5 however, differentiated the mesenchymal and epithelial phenotypes of bladder cancer cell lines in this panel. Indeed, the protein expression of PAK5 positively correlated with the protein expression profile of E-cadherin, and negatively correlated with N-cadherin in this panel of cell lines. To my knowledge, this is the first time this correlation has ever been described. This finding elicits the notion that PAK5 may have a novel role in bladder cancer differentiation and progression, and may interact with cadherin in the maintenance of epithelial morphology in papillary urothelial tumour.

My findings of high PAK5 expression in which correlated with epithelial morphology in bladder cancer cell lines may be contradictory to the previously described roles of PAK5 in invasion and metastasis, as suggested by studies done in the setting of colorectal (Gong, An et al. 2009, Wang, Gong et al. 2010), gastric (Gu, Li et al. 2013) and hepatocellular carcinoma (Fang, Jiang et al. 2014). On reviewing these publications, the sensitivity and specificity antibodies used to detect PAK5 could have contributed significantly to the conflicting result.

Firstly, in a study which showed that PAK5 was overexpressed during colorectal cancer progression and regulated colorectal carcinoma cell adhesion and migration (Gong, An et al. 2009), the isoform specificity of the polyclonal antibody used in this study for PAK5 had not been well described. The epitope for antibody recognition was generated against 224-amino acids N-terminal sequence of PAK5, and cross reaction with PAK4 had not been excluded in this paper. By Western blotting, the antibody was validated using Flag-PAK5, with myc-PAK1 and Flag PAK6, but not PAK4-fusion protein as negative controls. The molecular weight of the protein detected by the antibody in this study, hence referred to as PAK5, was smaller than 72kDa. The molecular

weight of ‘PAK5’ in the study was inconsistent with the molecular weight of PAK5 at 81kDa, as detected by my PAK5 specific antibody, and reported by other groups (Dan, Nath et al. 2002, Pandey, Dan et al. 2002, Cotteret and Chernoff 2006, Fang, Jiang et al. 2014). As PAK4 is structurally closely related to PAK5, and has the lowest MW (64 kDa) amongst the group 2 PAKs, cross reaction of this antibody with PAK4 on Western blot, indirect immunofluorescence and immunohistochemistry must to be excluded.

The validity of the antibody used in the study for the role of PAK5 in the development of gastric cancer (Gu, Li et al. 2013) was also unclear. The antibody, as published in the material section of this paper was a Mouse anti-PAK7 (PAK5) from Santa Cruz, Ca. In my work to screen for PAK5 expression in bladder cancer, I have used 2 PAK5 isoform-specific antibodies from Santa Cruz, and the available antibodies for PAK5 were both produced in goat (H-20 and S-16). The European representative for manufacturer, SCBT Europe was contacted, and confirmed that Santa Cruz did not have PAK7 antibody produced in mouse. In addition, this paper also showed distinct protein band for PAK5 in HEK293 cells on Western blotting, whereas previously published paper on PAK5 and SCBT product literature have reported that HEK293 cells did not express endogenous PAK5 ((Wu, Carr et al. 2008) and http://datasheets.scbt.com/sc-22155.pdf).

There are also other known characteristics of PAK5 which could have contributed to the possible contradiction. Its role may vary in different types of epithelia, or at different stages of malignant transformation. In developmental studies, the expression of PAK5 has been shown to be tissue-specific in mammalian physiology, being distributed predominantly in the brain, and at lower levels in several other tissues (Pandey, Dan et al. 2002, Li and Minden 2003). Whilst PAK5 was expressed in all regions of the brain, higher levels of expression ware detected in the cerebellum, cerebral cortex and olfactory bulb. However, when embryonic (E13) and adult brain were compared, it was noted that the expression of PAK5 in embryonic cerebellar primordium was negligible, in contrast to the high expression of PAK5 in the adult cerebellum, which suggests that PAK5 is preferentially expressed in mature neural systems (Pandey, Dan et al. 2002). This variation, observed in neuronal tissues in the context of developmental studies may also be a feature of PAK5 in oncogenesis.

In the following chapters, I continued to characterise the expression of PAK5 in bladder cancer cell lines, and investigated whether PAK5 has any direct role in the maintenance of epithelial morphology in RT4 and RT112.

Chapter 4 : Characterisation of PAK5 expression in bladder cancer

4.1 Introduction

In chapter 3, I have identified a distinct protein expression profile of PAK5 in bladder cancer cell lines, where higher expression levels were observed in the cancer cell lines with differentiated epithelial morphology. To date, the role of PAK5 in bladder cancer has not been well characterised, and further research into PAK5 were required to assess its utility as a potential biomarker for non-invasive bladder cancer.

In order to further understand the gene expression of PAK5 in bladder cancer, I screened the mRNA transcription profiles for PAK5 in patient-derived malignant and non-malignant bladder tissue specimen provided by Cardiff University collaborators. Publications and publicly available microarray data on Gene Expression Omnibus (GEO) were also searched for any information regarding the expression of PAK5 in bladder cancer. There has not been any peer-reviewed published data on PAK5 mRNA transcription in bladder cancer during the duration of my research. However, one high throughput genomic study (Dyrskjot, Kruhoffer et al. 2004) had included the gene expression of PAK5 in normal bladder tissue, and in urothelial tumours of different grades and stages (data could be accessed at http://www.ncbi.nlm.nih.gov/geoprofiles/14788769). The data on PAK5 had not been discussed in any publications by the authors from the group. I have therefore analysed the data, and the results were presented in sections of this chapter.

In addition to screening of patient samples, I have also profiled the mRNA transcription of PAK5 in bladder cancer cell lines in my panel. In this chapter, I presented my findings, where the profile mRNA transcription of PAK5 did not directly translate into protein expression in these cell lines. The postulations to explain this discrepancy were discussed at the end of this chapter.

At protein level, studies to investigate the regulatory mechanism and function of PAK5 had identified different regulatory inputs controlling the subcellular localisation of PAK5, hence its functions (Wu and Frost 2006, Wu, Carr et al. 2008). Multiple Rho proteins interact with PAK5, including Cdc42, Rac, RhoD and RhoH, which targeted PAK5 to specific locations within the cells. Amongst the Rho GTPases, PAK5 binds with greatest affinity to constitutively active Cdc42 ($Cdc42V^{12}$). Co-expression of WT PAK5 with $Cdc42V^{12}$ has been shown to substantially alter its

cellular localisation and directed WT PAK5 to filopodia within the plasma membrane, compared to overexpression of WT PAK5 alone which localised PAK5 to the mitochondria (Wu and Frost 2006). In addition to localisation to the mitochondria and the cell membrane, studies have also identified the cellular localisation of PAK5 to the nucleus, cytoplasm and centrosome.

Most of the studies that described the subcellular localisation of PAK5 were carried out in neuronal cells, or with PAK5 overexpression. In epithelial cell system, the subcellular localisation of endogenous PAK5 has not been well characterised. The distinct protein expression of PAK5 in bladder cancer cell lines RT4 and RT112, with differentiated epithelial morphology, had presented an opportunity to investigate the role of PAK5 in the urothelium specifically, which may be postulated to the epithelium in general.

In this chapter, I presented my findings of PAK5 localisation at cell-cell adherens junctions at steady state, and its internalisation in distinct vesicular cytoplasmic structures following stimulation with exogenous growth factor ligand. The effects of PAK5 downregulation by siRNA knockdown on the epithelial characteristic of RT4 cells were also described in this chapter.

4.2 Results

4.2.1 Reduction in PAK5 mRNA transcription was associated with urothelial carcinoma in human patient bladder tissue specimen of Cardiff patient cohort

The differential level of PAK5 protein expression in bladder cancer cell line strongly correlated with epithelial morphology. In order to investigate whether the expression level of PAK5 has the potential to be used as a biomarker, I have collaborated with Professor Wen Jiang and Dr Tracy Martin in at Cardiff University Hospital to access a small library of mRNA (reverse transcribed into cDNA for storage) from human bladder tissue samples. The screening for mRNA was identified as one of the suitable methods to screen for PAK5 expression in tissue samples at this point. Immunohistochemistry has not been done in this project as commercial antibodies for PAK5, previously tested in this lab failed to reliably detect PAK5. The in-house antibody I generated for PAK5 requires further assessment and validation for use in tissue immunostaining.

The cDNA library used consisted of 34 samples of tumour tissues and 18 samples from bladder tissues without any malignancy. In this experiment, I decided to screen for the expression of PAK1, PAK5 and PAK6. Even though PAK1 protein expression did not correlate with invasive potential when screened in bladder cancer cell lines, the mRNA expression of PAK1 has been previously studied in bladder cancer and was shown to be associated with increased risk of tumour recurrence (Ito, Nishiyama et al. 2007). The mRNA expression for PAK6 was also included in the screen due to the structural similarity, and possible functional redundancy with PAK5, as suggested by mouse PAK5/PAK6 targeted knockout study(Nekrasova, Jobes et al. 2008).

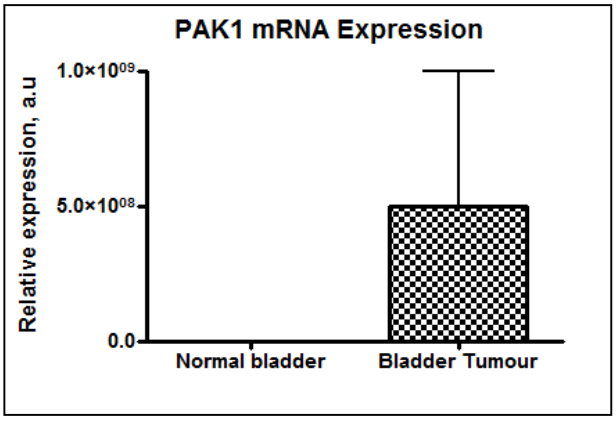
For quantitative analysis of PAK1, PAK5 and PAK6 transcripts, I employed a quantitative real-time polymerase chain reaction qRT PCR assay. The Ampliflor Uniprimer system was used as the probe system. The cDNA from the tissues were amplified simultaneously on an Icycler^{IQ5} system (Bio-Rad). The concentration of the respective transcript was calculated from the standard curve, which was simultaneously generated. The levels of the transcripts shown here are the ratios of respective PAK transcripts normalised to the level of GAPDH.

The values obtained from the assay indicated that in human patient samples, PAK1 and PAK6 transcripts were expressed at higher levels in malignant urothelium, compared to non-malignant epithelium (figure 4.1A & C). The expression of PAK5 mRNA however, was lower in malignant epithelium compared to non-malignant bladder tissue (figure 4.1B). It was noted that the mRNA expression obtained in this assay varied greatly between each individual samples, even those within the subgroup (malignant/non-malignant), as indicated by the error bar on the chart. Due to this variation, the differential expression of PAK1, PAK5 and PAK6 detected in this assay did not achieve any statistical significance.

The samples that I screened for PAK transcripts in this assay lacked the information for tumour grade, tumour stage or clinical follow-up outcomes. This has prevented me from doing any further subgroup analysis, especially in the subgroup with malignancy. I was therefore unable to interrogate whether differential expression could be detected between high grade and low grade tumours, as indicated by the differential expression of PAK5 protein seen in urothelial cancer cell lines.

Despite the limitations, the results of the screening of PAK5 in the Cardiff cohort of patients were consistent with the cell line findings for PAK5 protein level in bladder cancer cell lines. I therefore continued to test my hypothesis that the reduction of PAK5 in bladder cancer could be associated with epithelial to mesenchymal transition or urothelial carcinoma.

A



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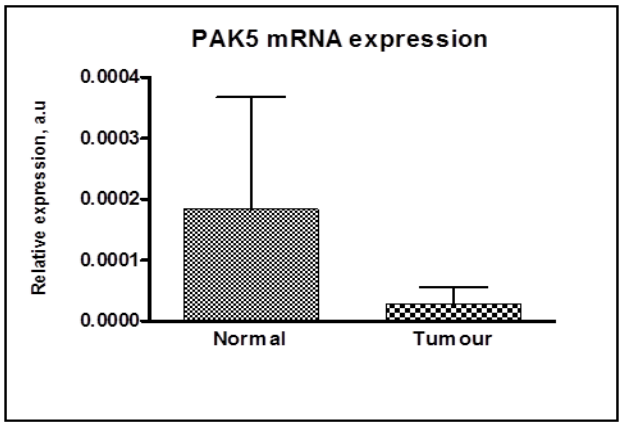

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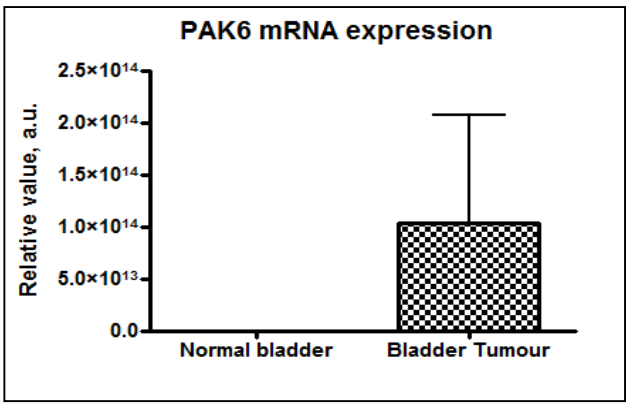


Figure 4-1 **(A-C): The mRNA expression level for PAK1, PAK5 and PAK6 in normal and malignant urothelial tissue samples.** The levels shown are the average (mean) values of PAK expression normalised to GAPDH. Standard error of the mean was calculated for all the samples included in the array. The results were not statistically significant (Student's *t*-test).

4.2.2 Reduction in PAK5 mRNA transcription was significantly linked to urothelial carcinoma in microarray data of human patient bladder tissue specimen on Gene Expression Omnibus

As the mRNA expression profile for PAK5 obtained from bladder tissue samples described in section 4.2.1 was underpowered, I searched for publicly available microarray data which included PAK5 expression in bladder cancer tissue on Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) to expand my studies. One set of data was available which included the mRNA expression of PAK5 (referred to as PAK7 in the search) in 60 bladder tissue specimen with complete histopathological staging and grading of the tumour. The microarray data was linked to a publication on carcinoma in situ (CIS) of the bladder (Dyrskjot, Kruhoffer et al. 2004), in which the association between PAK5 and CIS was not identified, or discussed.

The data in the microarray (GEO: GPL96, 213990_s_at (ID_REF), GDS1479, 57144) had been normalised using the Robust Multi-array Analysis (RMA). First, the data were analysed by comparing the mRNA transcription of PAK5 in normal urothelial specimen in this population (including normal urothelium obtained from patients with past history of urothelial cancer), and specimen from malignant urothelium of all grades and stages. PAK5 expression was higher in normal bladder tissue, and was downregulated in tumour tissues (figure 4.2A), similar to the finding of PAK5 mRNA expression in Cardiff cohort (figure 4.1). In addition, the difference was statistically significant (Student's *t*-test, ** = $P < 0.05$)

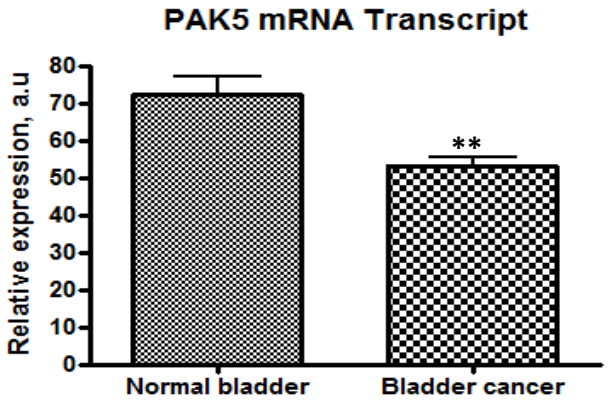
To further analyse of PAK5 expression in this set of samples, I have subdivided the tumours into the following 4 groups to indicate the risk of bladder cancer progression, and progressively poorer prognosis:

1. Normal bladder tissue
2. Low risk non-muscle invasive bladder tumours (stage: pTa, grade: 2, no CIS)
3. Moderate to high risk non-muscle invasive bladder tumours (stage pTa-1 grade 2 with CIS, CIS, all stage pTa-1 grade 3 tumours)
4. Muscle invasive bladder tumours (tumour stage T2 and above)

Analysis of PAK5 mRNA levels according to these subgroups also indicated that PAK5 mRNA transcription were lower in tumours of all subgroups, compared to normal bladder tissues (figure 4.2B). Statistical analysis comparing the expression levels between the 4 subgroups (one-way analysis of variance, ANOVA) detected significant difference in PAK5 mRNA expression in bladder cancer compared to normal tissue (figure 4.2B). However, no significant difference was detected in the expression of PAK5 between the low and high grade/stage bladder tumours.

The data for mRNA expression of PAK1 and PAK6 were also analysed to compare the findings in this microarray population to the Cardiff mRNA library previously screened. The expression profile for PAK1 did not indicate any association with risks of tumour progression (figure 4.3A). For PAK6, the average (mean) expression levels suggested that PAK6 may be upregulated as tumours progress from normal to muscle-invasive disease (figure 4.3B). However, one-way analysis of variance (ANOVA) did not detect any statistically significant difference for PAK6 mRNA transcription between any of the tumour groups compared to normal bladder tissue specimens in this microarray (figure 4.3C).

A



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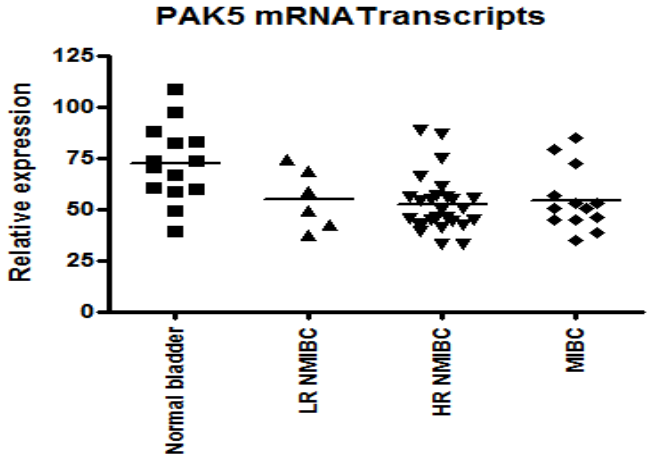
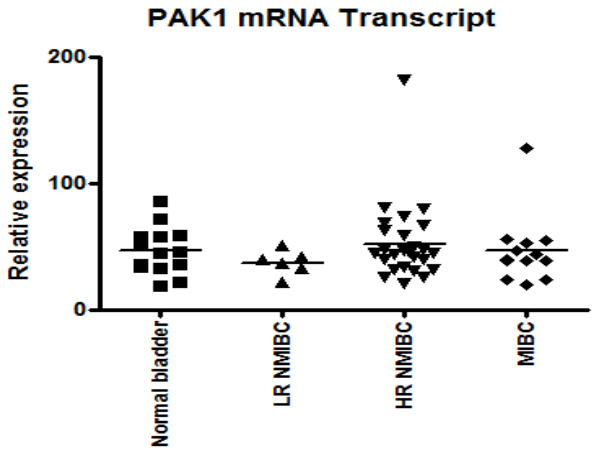


Statistical analysis	
One-way analysis of variance	
P value	0.0024
P value summary	**
Are means significantly different? (P < 0.05)	Yes
Number of groups	4

Figure 4-2 A): **Analysis for expression of PAK5 mRNA transcript in a microarray of 60 urothelial tissue specimen** A) mRNA expression of PAK5 in normal bladder tissue compared to bladder tumours. B) Micro array data for PAK5 mRNA were subdivided into groups for normal bladder tissue, low risk non-muscle invasive bladder cancer, (LR NMIBC), high risk non-muscle invasive bladder cancer, (LR NMIBC), and muscle invasive bladder cancer, (MIBC). **B)** Statistical analysis (ANOVA) for the difference in PAK5 mRNA transcription levels between the groups.

A



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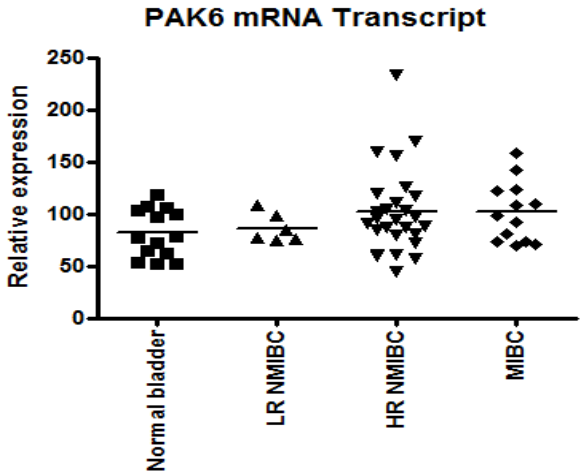

C

One-way analysis of variance		
	PAK1	PAK6
P value	0.6309	0.2377
P value summary	ns	Ns
P < 0.05	No	No
Number of groups	4	4

Figure 4-3 A) Expression of PAK1 mRNA transcript in a microarray of 60 urothelial tissue specimen subdivided into groups of normal bladder tissue, low risk non-muscle invasive bladder cancer, (LR NMIBC), high risk non-muscle invasive bladder cancer, (LR NMIBC), and muscle invasive bladder cancer, (MIBC). **B)** Statistical analysis (ANOVA) for PAK1 and PAK6 mRNA levels between the groups within the cohort.

4.2.3 PAK5 mRNA transcription did not show direct concordance with PAK5 protein levels in bladder cancer cell lines

To complement the profile for protein expression of PAK5 previously presented in chapter 3 (section 3.2.9, figure 3.15), I have also assessed the mRNA transcription profile of PAK5 in the panel of cell lines. The results of the following assay will enable further postulations on whether the differential protein expression profile of PAK5 seen in bladder cancer cell lines was a direct gene translation. If the results were discordant, it is possible that epigenetic factors or post-translational changes may also contribute to the distinct association between PAK5 and the epithelial differentiation of urothelial cancer cell lines.

The cells used in this assay were grown as monolayer in basal growth condition in 6-well plates for 24 hours before being harvested. Total RNA was extracted and purified from the cells, and then reverse transcribed into cDNA. Three sets of cDNA for each cell lines were prepared from 3 separate experiments were used for statistical analysis. The mRNA expression levels presented here had been normalised to the GAPDH ratio.

In this panel, RT112 showed the highest mRNA expression level for PAK5, whereas RT4, TCCSUP and 253J showed moderate level of mRNA expression. The lowest level of mRNA transcript for PAK5 was detected in T24, which also had the lowest protein expression of PAK5 in this panel of cell lines (figure 4.4). The mRNA expression profile in the cell lines differed to protein expression profile presented in the previous section (figure 3.14). It could be speculated that the translation of PAK5 may be downregulated in RT112, TCCSUP, and 253J, resulting in reduced total protein expression. Conversely, it could also be speculated that the protein levels of PAK5 in RT4 cells are more stable, and less dependent on the mRNA transcripts compared to the other cell lines.

Base on this result, great emphasis had to be placed on the quantification of PAK5 total protein as a conformational measure of PAK5 expression or downregulation in future experiments, as the mRNA transcription level for PAK5 may not reflect the protein expression affecting the phenotype(s) in bladder cancer cells.

A

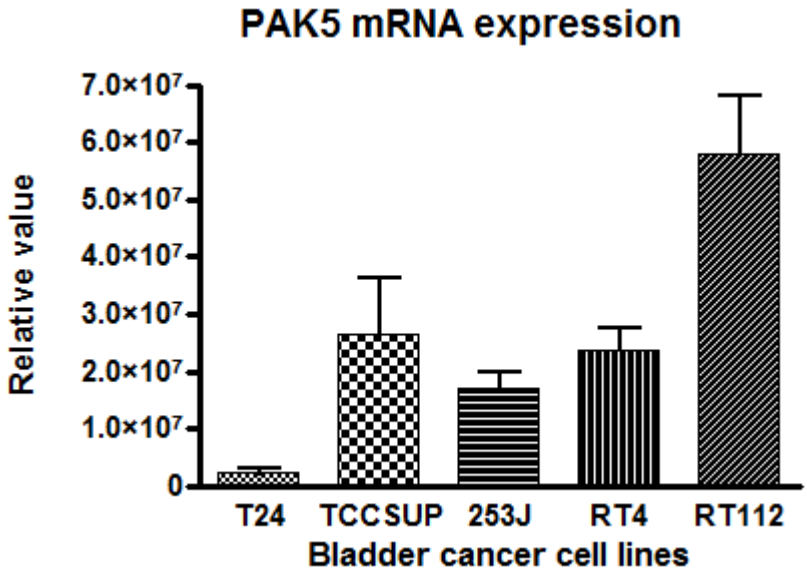

Figure 4-4**: The mRNA expression profile for PAK5.** Total mRNA for bladder cancer cell lines were extracted and reverse transcribed into cDNA for stability in storage. The values presented are the average (mean) PAK5 mRNA expression normalised to GAPDH from 3 independent experiments.

4.2.4 **Validation of PAK5 antibody for indirect immunofluorescence**

The role of PAK5 in urothelial tumour had not been previously characterised. Following my novel finding of high PAK5 protein expression in RT4 and RT112 cells, I sought to study the role of PAK5 in these cell lines. A validated PAK5 antibody that works on immunofluorescence would provide a valuable tool to characterise endogenous PAK5 expressed in these cells. I therefore proceeded to demonstrate the utility of the in-house antibody for the detection of PAK5 in immunofluorescent studies.

The antibody was validated by showing that it detected GFP-fusion PAK5 protein over expressed in cell lines without endogenous protein expression for PAK5. T24 cells demonstrated the lowest mRNA expression level for PAK5, which corresponded with undetectable protein expression on Western blot, had provided a good model for the validation. In this experiment, T24 cells were seeded on glass coverslips and transfected with GFP-PAK5 cDNA using Xtremegene HP according to manufacturer's protocol. Cells on coverslips were fixed with 4% PFA, and stained with PAK5 in-house antibody (1:400 in 3% BSA: PBS solution) for indirect immunofluorescent as previously described. T24 cells transfected with GFP-empty vector was used as negative control.

The in-house PAK5 antibody detected GFP-PAK5, but did not detect any proteins/subcellular structures in T24 cells over-expressing GFP-alone (figure 4.5). In T24 cells, I noted distinct GFP-PAK5 overexpression in punctate subcellular structures, excluded from the nucleus. Over-expressed GFP-PAK5 was also detected at lower level in the diffuse cytoplasmic background. These findings were consistent with previously reported observations ((Matenia, Griesshaber et al. 2005, Cotteret and Chernoff 2006, Wu and Frost 2006), over expression of PAK5 (WT).

The findings of this validation experiment indicated that the in-house PAK5 antibody had the potential to sensitively and specifically detect endogenous PAK5 by immunofluorescence in RT4 and RT112 cells.

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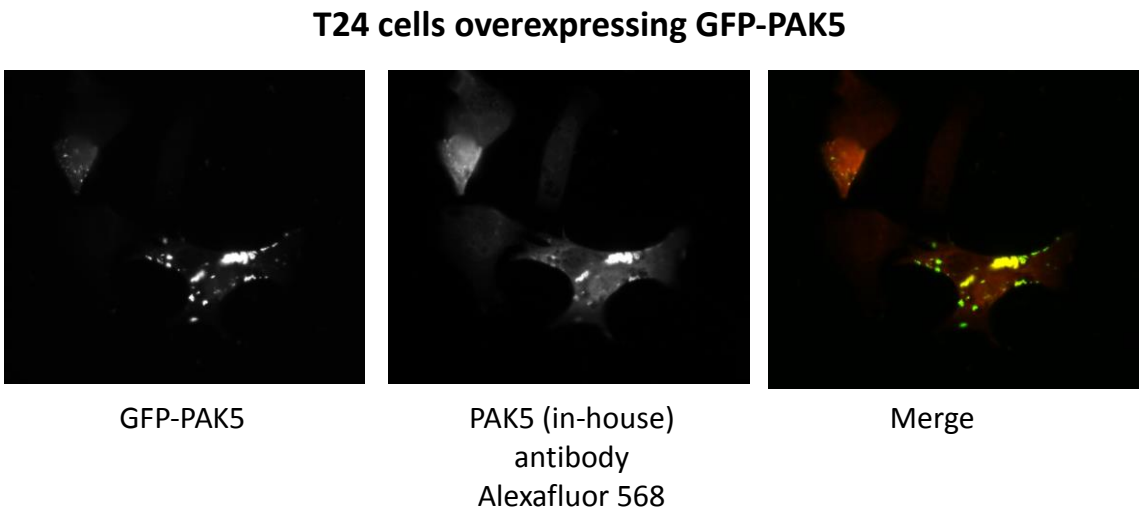


B

Figure 4-5 **: Validation of in-house PAK5 antibody**. A) GFP-PAK5 was overexpressed in T24 cells and immunolabelled with PAK5 in-house antibody conjugated to Alexafluor 568. Images were merged to emphasise sensitivity of the antibody. B) T24 cells overexpressing GFP-empty vector was used as negative control.

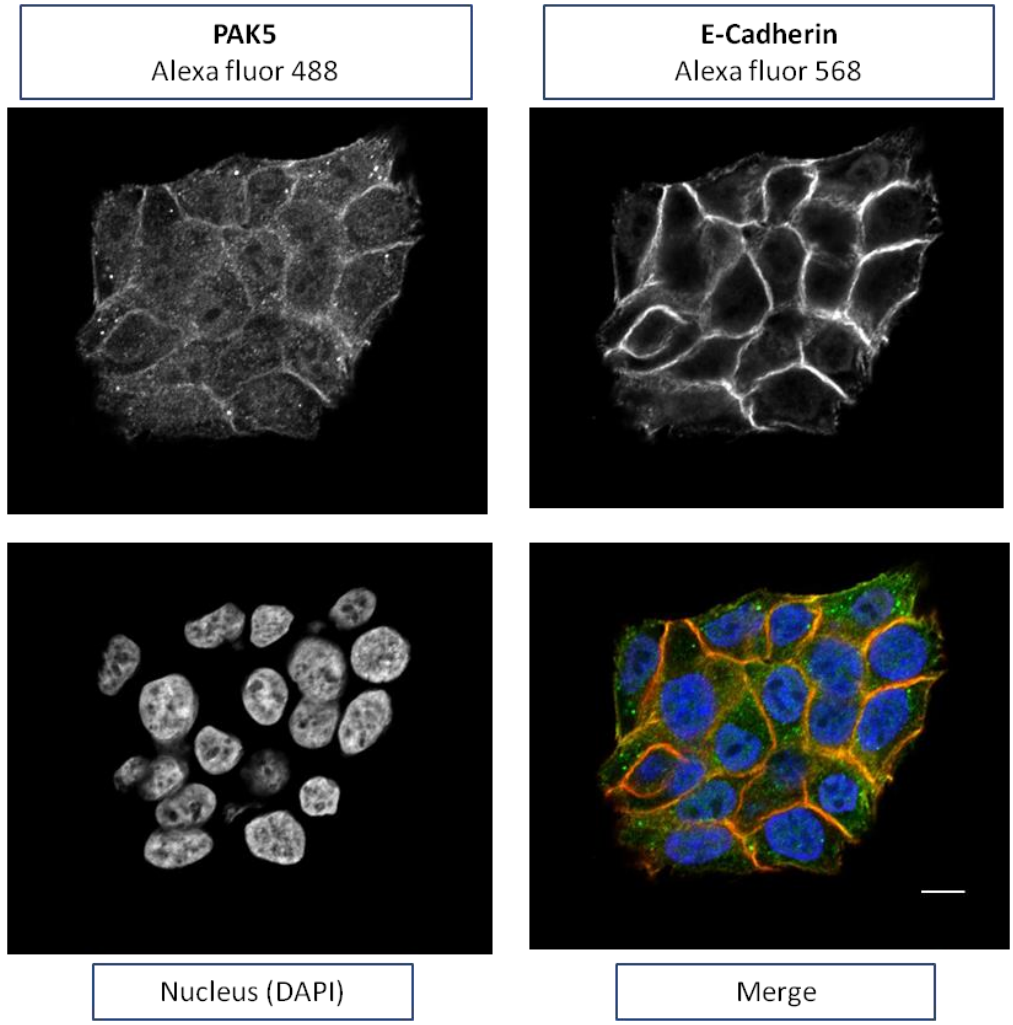
4.2.5 Subcellular localisation of endogenous PAK5 in RT112 and RT4 cells under basal growth conditions

There is substantial level of evidence to demonstrate that PAK5 is spatially regulated. Studies had presented the evidence that the regulatory N-terminal domain of PAK5 contained functional sequences that regulate the intracellular localisation, and this localisation was critical to the biological function of PAK5 (Cotteret, Jaffer et al. 2003, Cotteret and Chernoff 2006). Interaction of PAK5 with different Rho GTPases, Cdc42 or RhoD via the CRIB domain had also been shown to direct PAK5 to specific subcellular localisations (Wu and Frost 2006). Interaction between PAK5 and constitutively active RhoD (V^{12}) directed its localisation to the mitochondria. Mitochondrial localisation of PAK5 was necessary for the regulation of its anti-apoptotic property via phosphorylation of the downstream substrate BAD (Cotteret and Chernoff 2006). In contrast, co-expression of PAK5 with constitutively active Cdc42 (V^{12}) directed its localisation substantially to the filopodia within the plasma membrane, and in punctate distribution excluding the mitochondria (Wu and Frost 2006). The functional role or downstream target for PAK5 at the cell membrane, or the significance of the punctae containing PAK5 and Cdc42 had not been characterised.

Using the in-house PAK5 antibody which I had validated in the previous section for indirect immunofluorescence, the expression of endogenous PAK5 in RT4 and RT112 cells were imaged. In RT4 cells (figure 4.6A), endogenous PAK5 protein was distributed in the cytoplasm in vesicular or punctate structures. In addition, distinct localisation of PAK5 in the membrane at the margins of cell-cell adhesion was noted. Co-staining of RT4 cells with E-Cadherin (HECD1) antibody detected co-localisation of PAK5 with E-cadherin at the cell-cell junctions, further analysed by line scanning of confocal images of the cells (figure 4.6B).

As previously presented in chapter 3, the adherens junctions in RT112 were less mature (figures 3.6), and the total expression of E-cadherin and PAK5 proteins were lower (figures 3.5 and 3.14) in RT112 compared to RT4 cells. Immunofluorescent staining for PAK5 in RT112 cells showed that endogenous PAK5 was also distributed in punctate structures in the cytoplasm (figure 4.7A). At the cell membrane, the expression of PAK5 colocalised with E-cadherin at margins of cell-cell adhesions (figure 4.7B).

A



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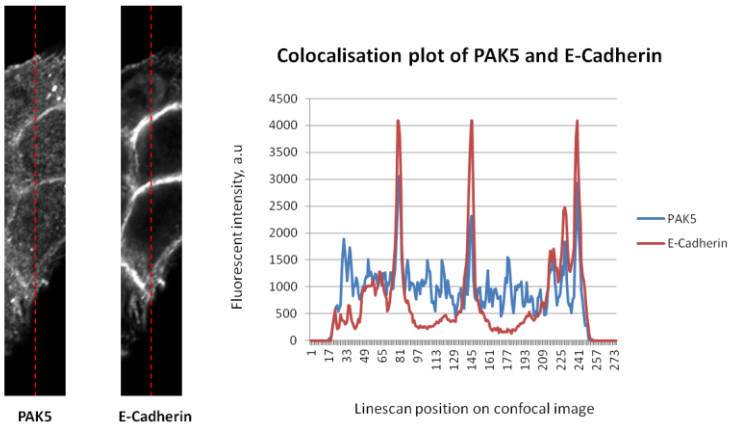
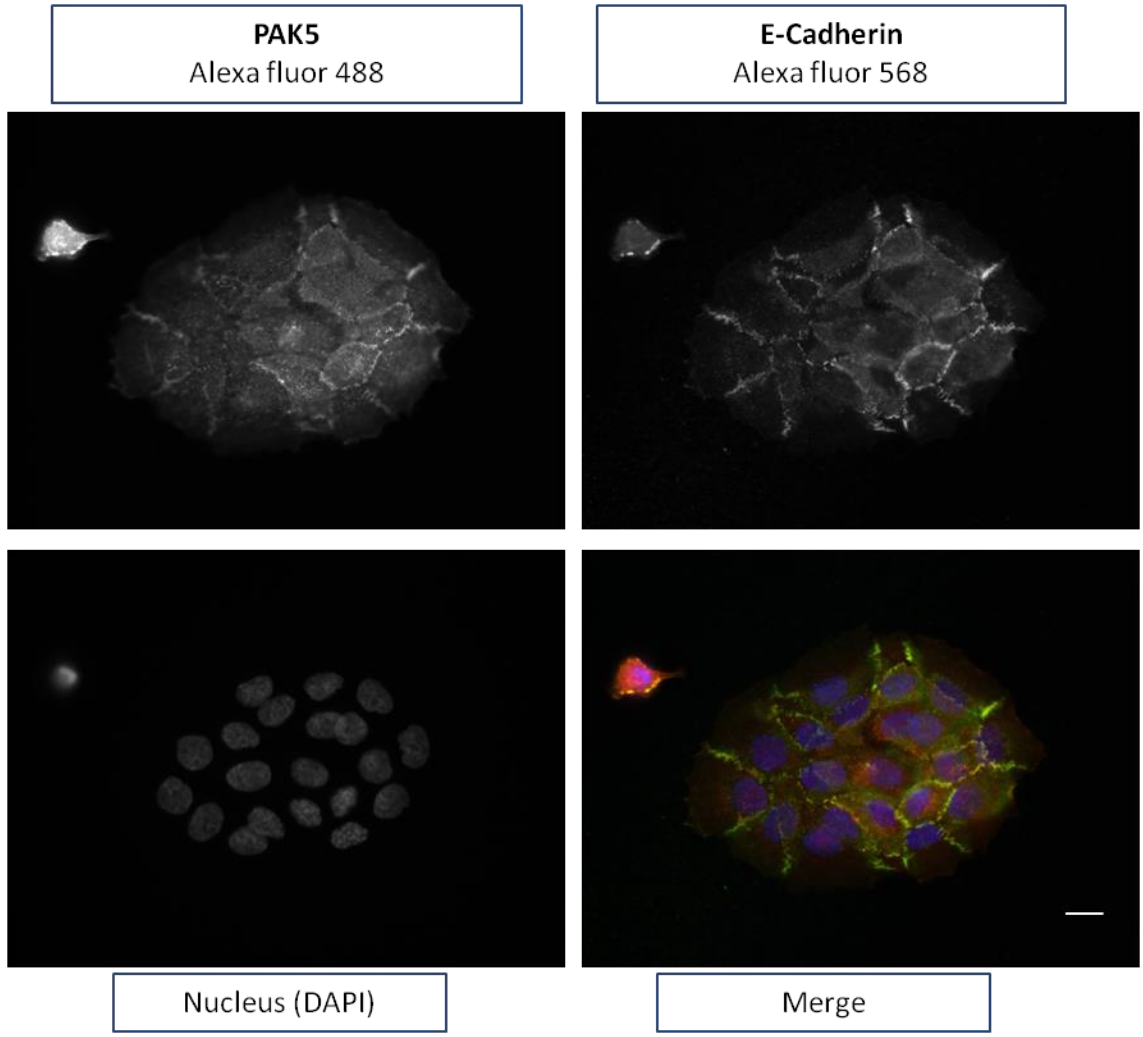


Figure 4-6 **: Subcellular localisation of endogenous PAK5 in RT4 cells.** Indirect immunofluorescent confocal microscopy images of RT4 cells at basal growth condition, co-probed with PAK5 and E-cadherin antibodies. Nuclei were stained with DAPI. Bar = 10μm.

A



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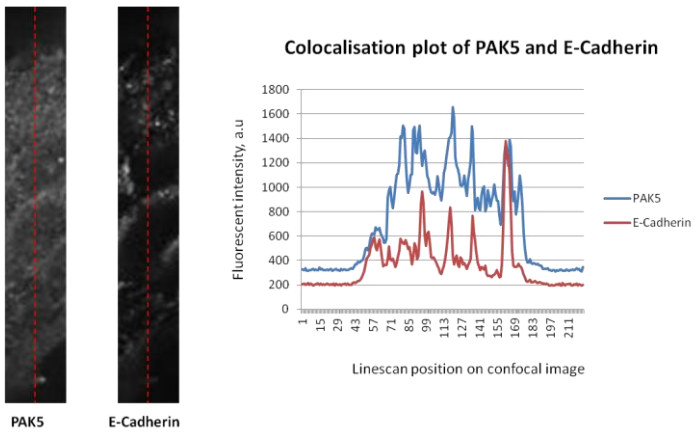


Figure 4-7 **: Subcellular localisation of endogenous PAK5 in RT112 cells.** Indirect immunofluorescent images of RT112 cells at basal growth condition, co-probed with PAK5 and E-cadherin antibodies. Nuclei were stained with DAPI. Bar = 10μm.

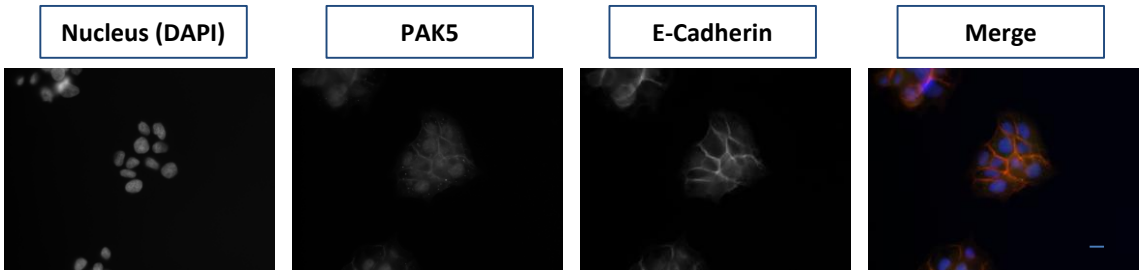
4.2.6 PAK5 also colocalised with internalised E-Cadherin following growth factor stimulation in RT4 cells

I have previously shown that in RT4 cells, E-cadherin colocalised with PAK5 predominantly at the cell-cell junctions at steady state (section 4.2.5, figure 4.6). Signalling by tyrosine kinases such as Hepatocyte Growth Factor (HGF) has been demonstrated to regulate the intracellular trafficking of E-Cadherin, where exogenous HGF stimulation of epithelial cells resulted in increased translocation and redistribution of E-cadherin from the cell membrane into the cytoplasm ((Kamei, Matozaki et al. 1999, Miura, Nishimura et al. 2001, Fujita, Krause et al. 2002, Lu, Ghosh et al. 2003, Bryant, Kerr et al. 2007). I have also demonstrated that HGF stimulation could affect the stability of cell-cell adherens junctions in RT4 cells by redistributing E-cadherin away from the adhesion plaque at the cell membrane (section 3.2.3, figure 3.9).

Growth factor signalling had also been demonstrated to regulate the subcellular localisation of some members of PAK-family kinases. HGF stimulation of MDCK cells resulted in the translocation of PAK1 to the membrane ruffles (Royal, Lamarche-Vane et al. 2000), but redistribution of PAK5 following HGF stimulation had not been reported. Stimulation of C17.2 neuronal cells by EGF had been described, which triggered PAK5 shuttling from the cytoplasm to the nucleus (Cotteret and Chernoff 2006). However, the role of growth factors in the subcellular redistribution of PAK5 in epithelial cells had not been characterised.

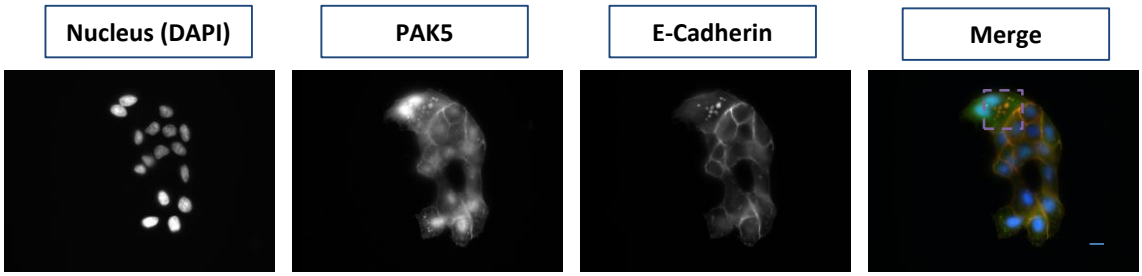
In this section I describe my findings on the subcellular localisation of E-Cadherin and PAK5 following stimulation with HGF. In control conditions, E-cadherin distribution was localised at the plaques of cell-cell adherens junctions, whilst PAK5 was distributed at the adherens junctions as well as in vesicular/punctate structures in the cytoplasm (figure 5.7A). Following HGF stimulation, E-Cadherin were redistributed away from the cell-cell adhesion plaques, and in a proportion of cells, accumulated in distinct, large vesicular structures (figure 4.6B & C). Accumulation of PAK5 was also observed in these E-cadherin enriched vesicles. A number of these vesicles were larger than 0.2μM in diameter, and appeared lobulated or tubulated (figure 4.6C).Line scan analysis of confocal images of PAK5 and E-Cadherin staining in RT4 cells were performed to confirm the colocalisation of these proteins in the vesicular structures following HGF-stimulation (figure 5.8A). The accumulation of E-Cadherin and PAK5 in the vesicular structures at 30 minutes of HGF-stimulation also coincided with maximal MAPK pathway activation in RT4 cells (figure 4.8B)

A



Serum-starved

B



HGF-stimulated

C

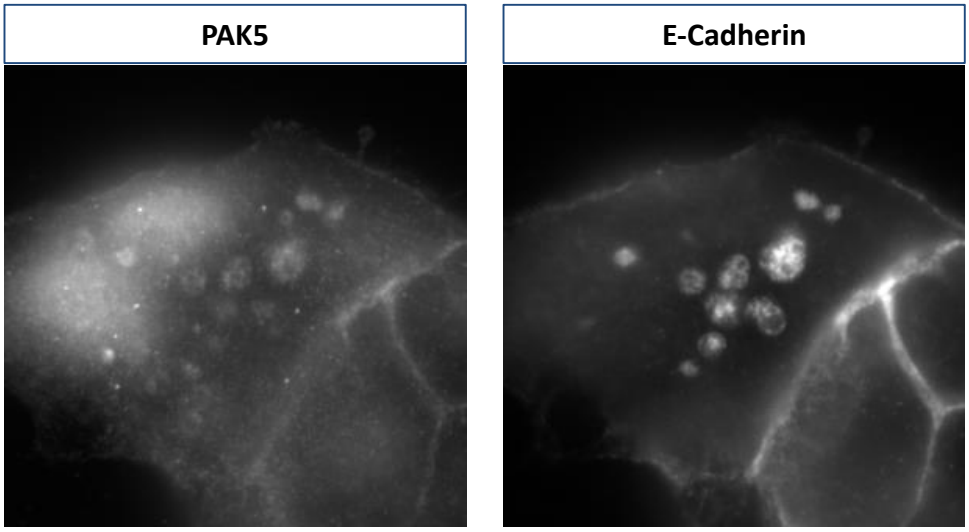
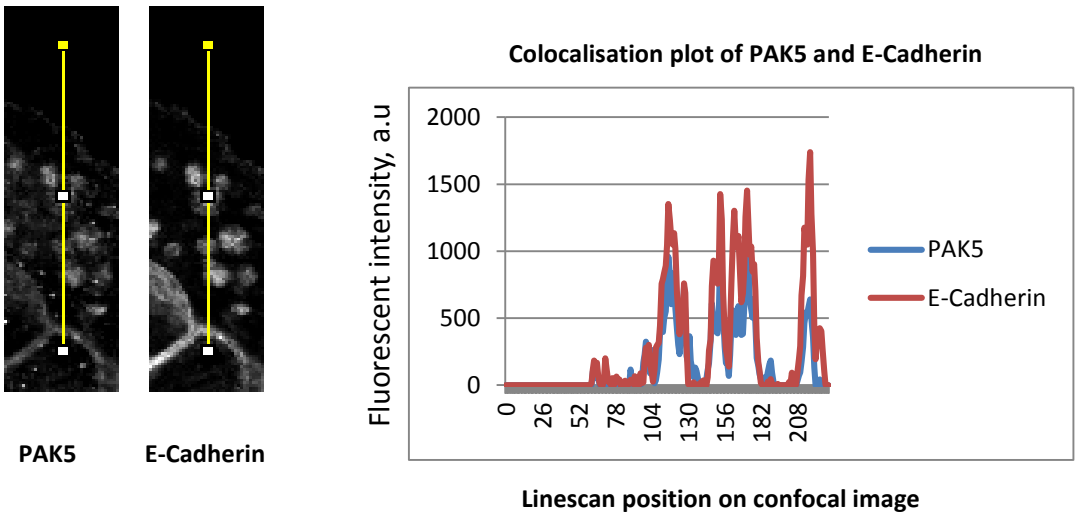


Figure 4-8 **: Subcellular localisation of PAK5 in RT4 cells following HGF stimulation** A) RT4 cells in serum-starved conditions stained for nucleus, PAK5 and E-Cadherin. B) RT4 cells in following 30 minutes of HGF stimulation stained for nucleus, PAK5 and E-Cadherin. Boxed area in the merged image indicates the area magnified in figure (C), where the boxed area included the vesicular and membranous distribution of PAK5 and E-Cadherin in HGF-stimulated condition.

A



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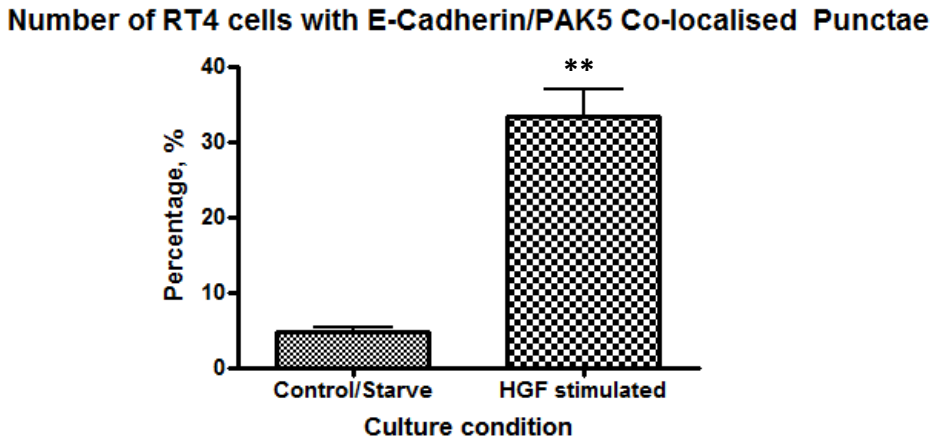


Figure 4-9 : **HGF stimulation of RT4 cells.** A) Co-localisation of PAK5 with E-cadherin in cytoplasmic vesicular structures in RT4 cells at 30-minutes of HGF-stimulation. Line-scans across the cytoplasmic vesicular structures of corresponding confocal images were performed, and the fluorescent intensities were quantified. The overlapping intensity peaks indicated co-localisation. B) Effects of HGF-stimulation on E-Cadherin/PAK5 colocalisation in cytoplasmic punctate. For HGF-stimulation, cells were fixed after 30 minutes following addition of HGF to serum-starved media. Values presented are the average (mean) representation of 50 cells from 3 independent experiments. Statistical significance compared to unstimulated/starve condition was calculated using Student's *t*-test; **, P, 0.05.

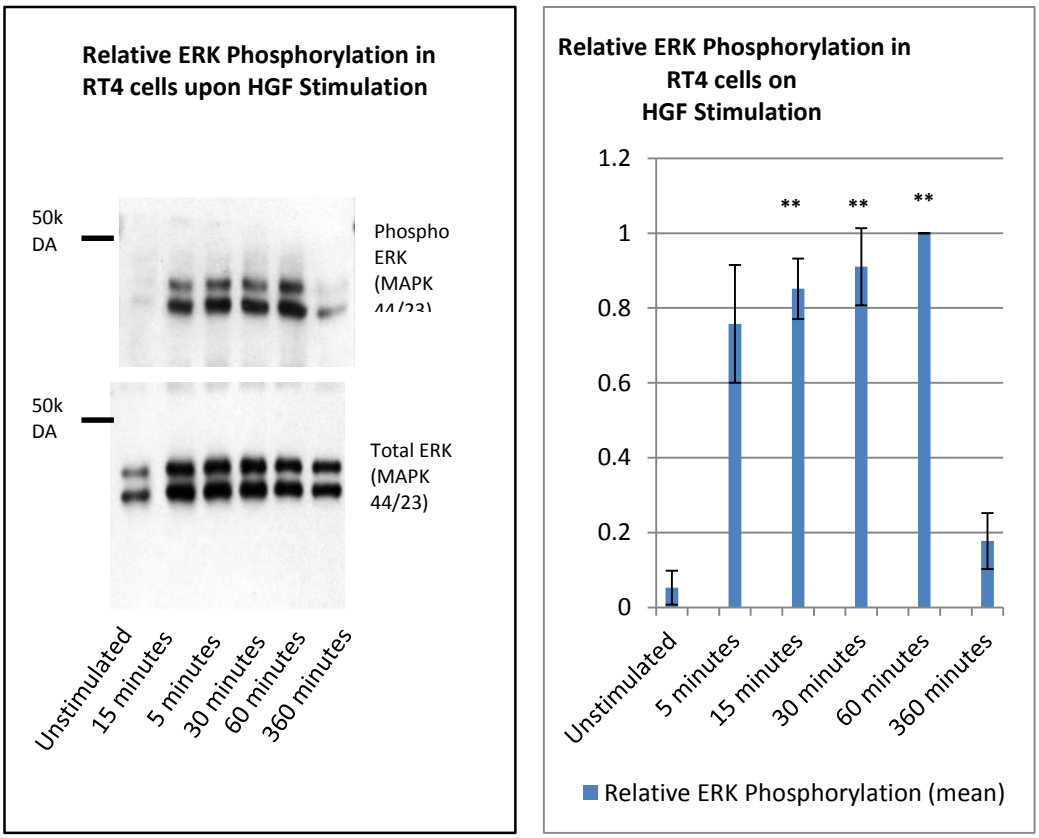


Figure 4-10 : **Relative ERK phosphorylation of RT4 cells upon HGF stimulation**. Whole cell lysates of RT4 cells at different time-points of HGF-stimulation were assayed for ERK/MAPK activation. The levels of phosphorylated ERK was normalised relative to the total ERK levels. Relative expressions quantified represent the mean of 3 independent experiments.

4.2.7 **Downregulation of PAK5 in RT4 cells by siRNA knockdown**

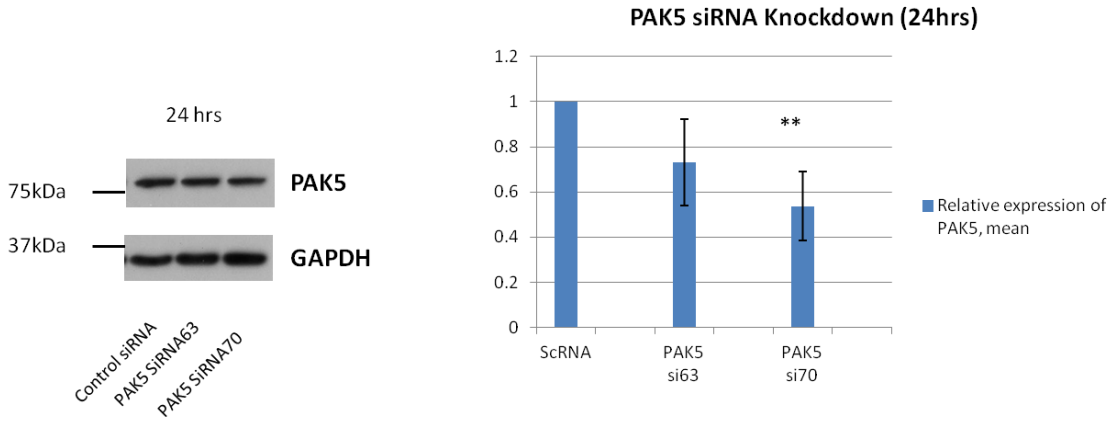
The high level of protein expression of PAK5, and its distinct colocalisation with E-cadherin at the cell-cell adhesions in RT4 cells presented it as a candidate as a model to study the role of PAK5 in epithelial differentiation of bladder cancer. As previously presented and discussed in chapter 3, very low or undetectable protein expression of PAK5 was detected in poorly differentiated bladder cancer cells such as T24, TCCSUP and 253J. I therefore hypothesised that PAK5 contributes towards the maintenance of cell-cell adherens junctions, and investigated the effects of PAK5 down-regulation in RT4 cells.

In order to downregulate PAK5 expression, I transiently transfected RT4 cells with PAK5 specific siRNA. Two different siRNA oligonucleotides were used for PAK5 gene silencing: Si63 and Si70. Oligonucleotide Si63 targeted PAK5 DNA in the coding region, whereas si70 targeted the 3' untranslated region (UTR). In order to identify the dynamics of PAK5 expression following transient siRNA transfection, the total protein expressions of PAK5 at 24, 48 and 72 hours post transfection were assessed and quantified by Western blotting.

At 24 hours (figure 4.6A), using PAK5 siRNA63 and siRNA70, the protein expression of PAK5 was downregulated by 25% and 50% respectively, compared to the control experiment. The maximal downregulation of PAK5 protein expression was observed at 48 hours following transient transfection (figure 4.6B), where the average protein expression levels were reduced by 60% by both PAK5 siRNA63 and siRNA70. At 72 hours, the protein expression levels for PAK5 recovered in RT4 cells transfected with siRNA63, with no significant difference detected for cells transfected with Si63 or control SiRNA at this time point (figure 4.6C).

The optimisation of PAK5 siRNA transfection in RT4 was a challenge due to the combination of these 2 reasons; RT4 cells could not be transfected efficiently, and PAK5 protein expression was only transiently downregulated within a short window at approximately 48 hours post transfection. These limitations could restrict further functional studies on the effects of PAK5 downregulation beyond 48 hours. Based on these results, I concluded that the downregulation of PAK5 expression in RT4 cells was most efficient at 48 hours when both PAK5 siRNA oligonucleotides were deployed to study the effects of PAK5 down regulation on the morphology of RT4 cells in the next section.

A



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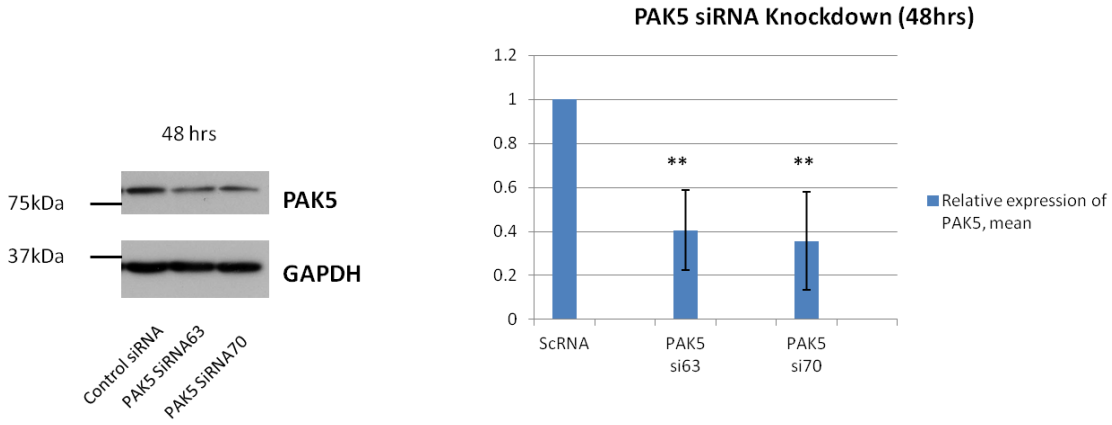

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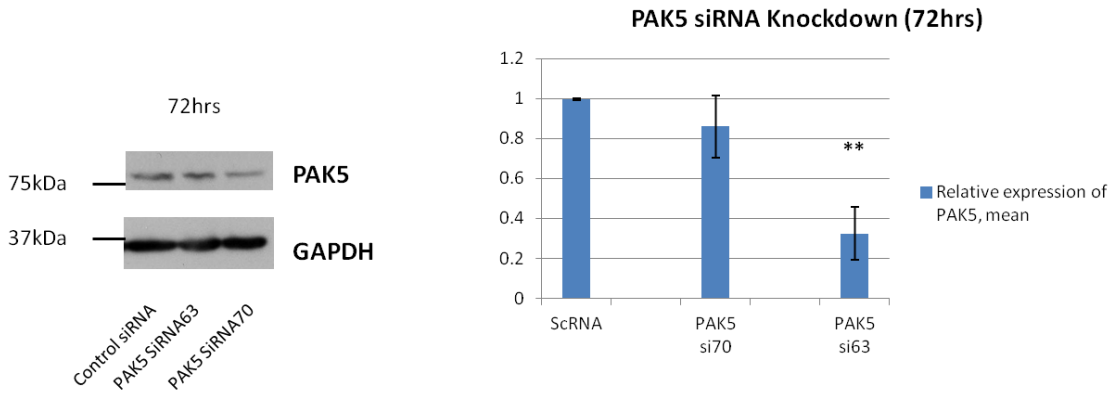


Figure 4-11 **: Optimisation of transient downregulation of PAK5 protein expression by siRNA knockdown.** Western blots for total protein levels of PAK5 and quantification of relative expression normalised to GAPDH at; A) 24 hours, B) 48 hours, and C)72 hours of siRNA transfection. Data represent the mean of 3 independent experiments. Statistical significance compared with Scrambled SiRNA (ScRNA) was calculated using Student's *t*-test; **, P, 0.05

4.2.8 Morphological changes in RT4 cells following PAK5 SiRNA knockdown

As demonstrated in the previous section, maximal downregulation of PAK5 protein level by siRNA knockdown was achieved at 48 hours of transient transfection. The morphological changes of the cells following PAK5 downregulation were therefore assessed in 2-D by immunofluorescence at this timepoint. The changes to the cell morphology and cell-cell adherens junctions following 48hours of transient transfection with PAK5 siRNA63 and siRNA70 were compared to control RT4 cells (control siRNA transfection).

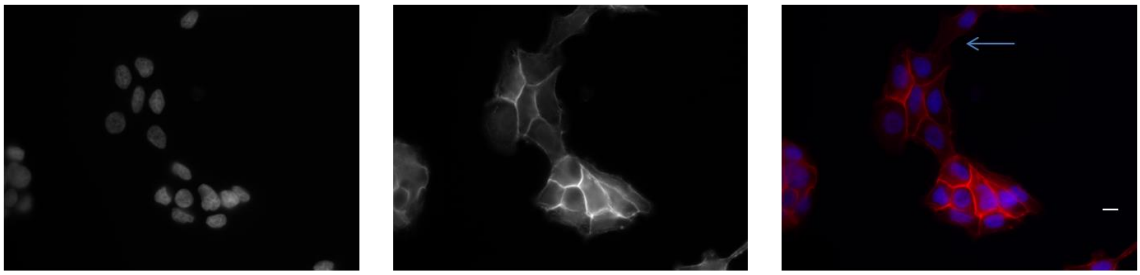
RT4 cells transfected with control siRNA maintained the cobblestone-like appearance within the epithelial colonies, with minimal difference in cell morphology irrespective of the number of cells in-contact with one another within the cell-colony (figure 4.7A). RT4 cells transfected with PAK5 siRNA63 and siRNA70 however appeared more rounded, with some cells showing varying stages of dissociation at the cell-cell adherens junctions (figure 4.7B, C).The changes to cell morphology and cell-cell adherens junctions were more pronounced when PAK5 was downregulated by si70, rather than si63, consistent with the efficiency of total PAK5 protein downregulation on Western blot presented in the previous section.

The heterogeneity of the morphological changes was also affected by the size of the cell colony, and whether the cells were in completely surrounded by other cells (circumferential contact with adjacent cells).

In order to quantify the state of cell-cell adhesion in RT4 following transient PAK5 siRNA knockdown, the model of cell-cell adhesion formation and maturation in 3 different stages (Adams, Chen et al. 1998, Vasioukhin, Bauer et al. 2000, Vasioukhin and Fuchs 2001) was used as a working concept. Briefly, cells with <50% of the membrane surface forming cell-cell contact with adjacent cells were considered to have less mature epithelial junctional formation and classified as ‘dissociating’ (diagram in figure 4.8A). The percentage, (%) of cells demonstrating ‘junctional dissociation’ as presented in figure 4.8B were calculated based on the following algorithm: number of cells with <50% (membrane) cell-cell contact /total number of cells x 100.

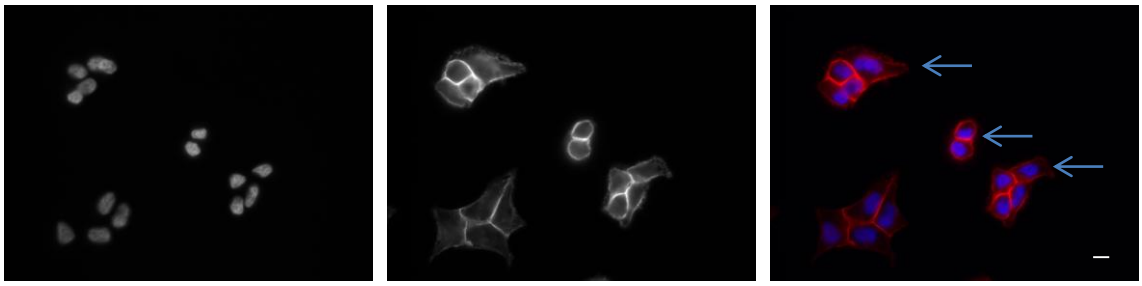
As the RT4 cells assessed in this assay for control and PAK5 siRNA were in small colonies instead of large epithelial sheets, a small proportion (15%) of RT4 cells in control experiment also demonstrated <50% membranous adherens cell-cell contact (indicated by arrow in figure 4.7A, quantified in figure 4.8B). Following PAK5 siRNA knockdown, significant proportion of RT4 cells (45% for siRNA63 and 60% for siRNA70) demonstrated <50% membranous cell-cell contact, classified as undergoing junctional dissociation (figure 4.8B).

A



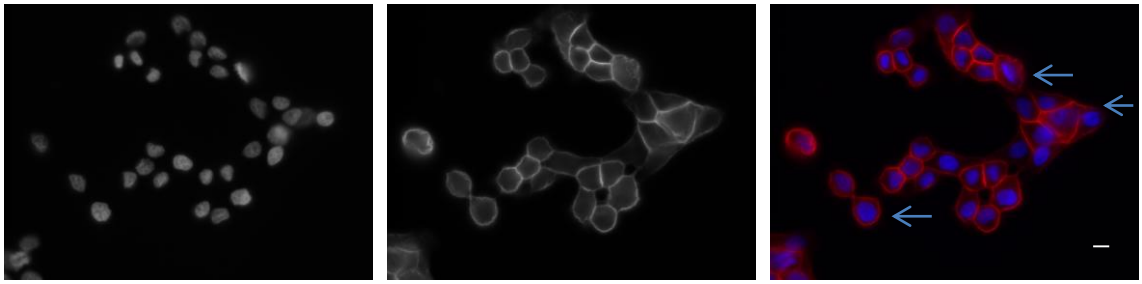
DAPI E-Cadherin Merge

B



DAPI E-Cadherin Merge

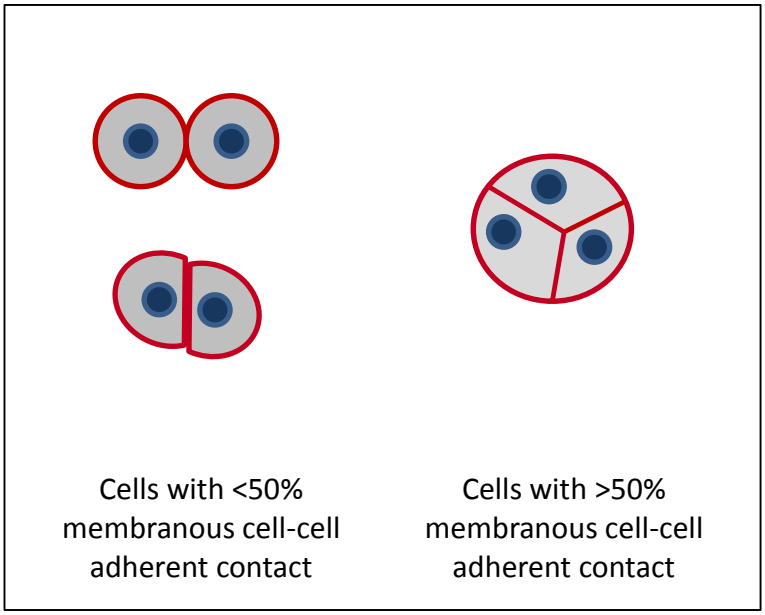
C



DAPI E-Cadherin Merge

Figure 4-12 **: Representative images of RT4 cells at 48 hours of transfection** with **(A)** control siRNA, **(B)** PAK5 siRNA63 and **(C)** siRNA70. Cells were stained for nucleus (DAPI) and E-cadherin. Arrows indicated cells with <50 cell-cell adherent contact. Bar = 10μm

A



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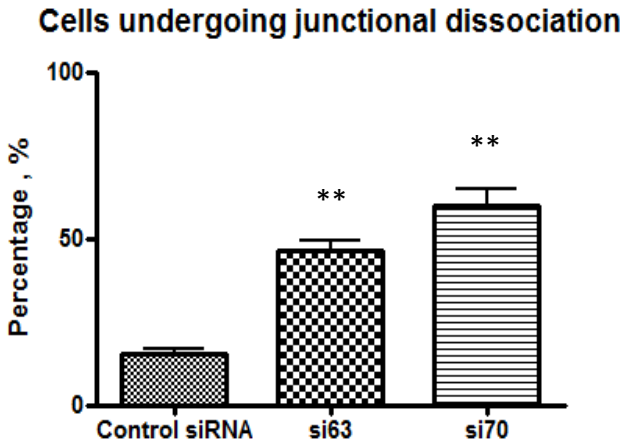


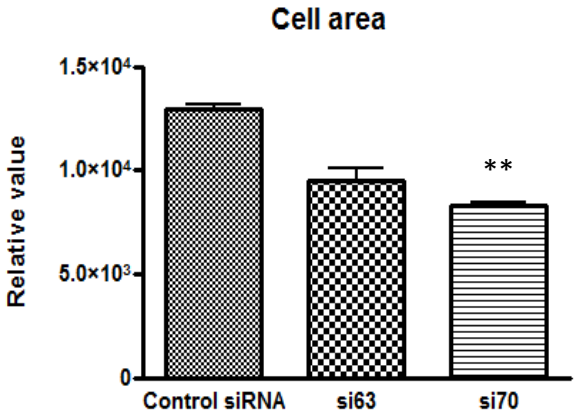
Figure 4-13 : **Quantification for cell-cell dissociation in RT4 cells following transient transfection with PAK5 siRNA.** A) Schematic representation of RT4 cells with established epithelial cell-cell adherens junctions (>50% cell-cell contact) and immature or dissociating junctions (<50% cell-cell contact). B) Quantification for the number of RT4 cells without established (mature) cell-cell junctions over the total number of cells. Quantification was performed on at least 50 cells per condition. The values presented are the average (mean) quantifications from 3 independent experiments; error bars indicate standard deviation of the means. Statistical significance for the difference compared to control siRNA was calculated using Student's *t*-test, **, $P < 0.05$.

4.2.9 **Cell-shape analysis of RT4 cells following transient PAK5 knockdown**

Cell shape in 2-D as indicated by the F-actin staining of the cell membrane, was analysed using imageJ software using set measurements, which included of area (relative value) and shape descriptors (circularity and aspect ratio). The morphological chances as indicated by cell-shape in RT4 cells transiently transfected with PAK5 siRNA oligonucleotides (siRNA63 and siRNA70) was analysed, and compared to the cell-shape of RT4 cells transfected with control siRNA.

The cell area measured indicated that RT4 cells with PAK5 knockdown were smaller compared to the control RT4 cells. The reduction in size following PAK5 downregulation was statistically significant following transfection using siRNA70, but not siRNA63 (figure 4.9A). The circularity, calculated using the formula; *circularity = 4pi (area/perimeter^2)* was enhanced in RT4 cells following PAK5 siRNA knockdown, with statistically significant difference compared to control cells for both PAK5 siRNA oligonucleotides (figure 4.9B) . Consistent with the increase in cell circularity, the aspect ratio of RT4 cells was reduced by PAK5 downregulation, with statistically significant difference compared to control for both siRNA63 and siRNA70 (figure 4.9C) .

A



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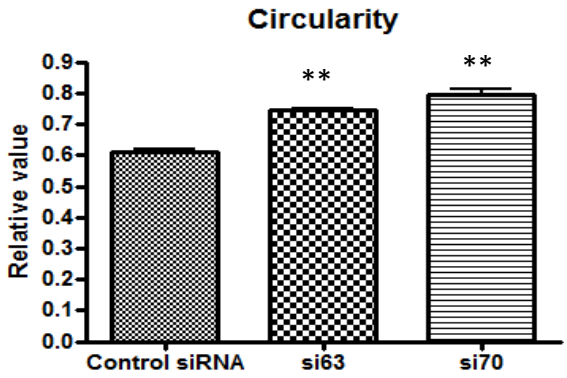

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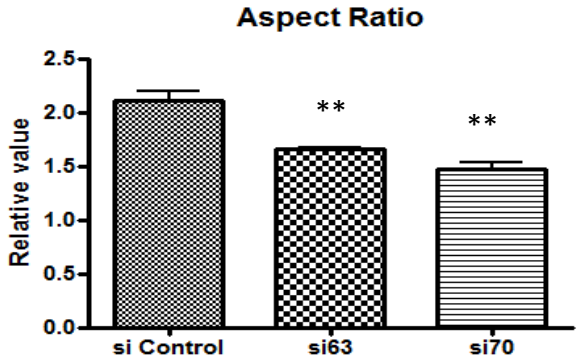


Figure 4-14 : **Cell shape analysis of RT4 following PAK5 siRNA knockdown.** A) Area. B) Circularity. C) Aspect ratio. The values presented are the mean values and standard error of the mean calculated from 3 independent experiments (50 cells analysed per condition/experiment). Statistical significance for the difference in morphological parameters of each PAK5 siRNA compared to control siRNA was calculated using Student's *t*-test; ** = $P < 0.05$

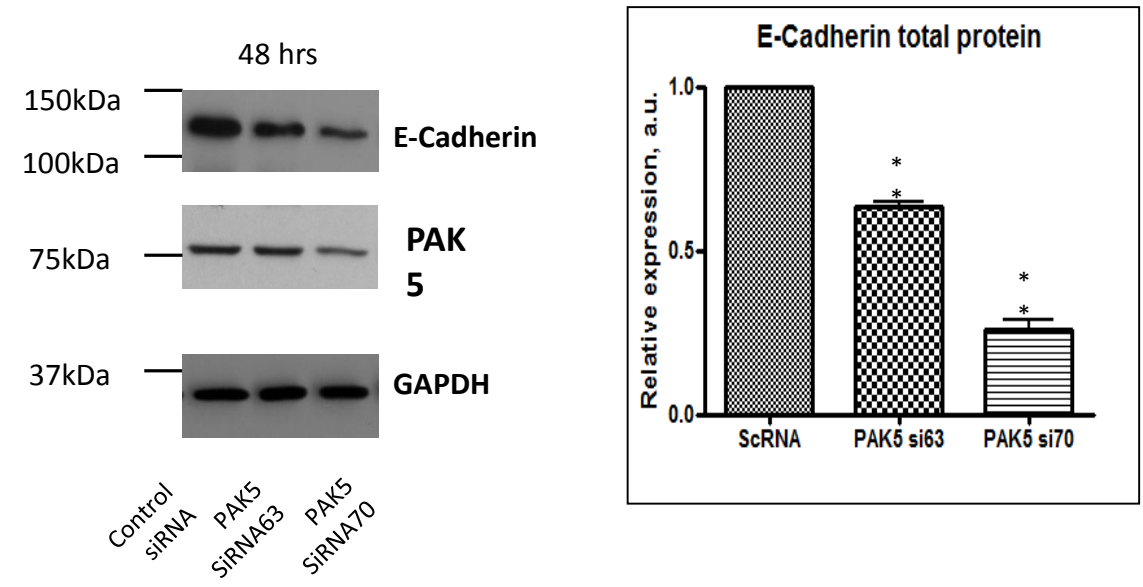
4.2.10 Downregulation of total E-cadherin and P120-catenin associated with PAK5 siRNA knockdown

The epithelial adherens junction is an E-cadherin-based complex that controls tissue integrity, and is stabilised at the plasma membrane by p120-catenin (Nelson and Nusse 2004, Schackmann, Tenhagen et al. 2013). In order to understand the mechanism for the dissociation of cell-cell junctions which occurred as a result of PAK5 siRNA knockdown, the fates of E-cadherin and p120-catenin were investigated. I thus assayed the total protein expression of both E-cadherin and p120-catenin in RT4 cells at 48 hours of PAK5 siRNA knockdown by western blotting.

The total protein expression for both E-Cadherin and P120-catenin were both downregulated following PAK5 silencing (figure 4.12). Consistent with the maximal PAK5 knockdown efficiency and maximal cell-cell dissociation index achieved by oligonucleotides siRNA70, the maximal downregulation of E-cadherin and P120-catenin protein levels were also attained by siRNA70. The down regulation was very prominent for P120-catenin (figure 4.12B). The antibody used in this assay has been reported to interact with all isoforms of p120-Catenin, with molecular weights ranging from 90-115kDa, which could have exaggerated the quantification accounting for the reduction of p120-Catenin levels at 48 hours of PAK5 knockdown compared to the E-Cadherin levels.

Although PAK5 downregulation could potentially affect the phosphorylation states of serine/threonine residues of E-Cadherin or p120-Catenin, I had not investigated these further due to the downregulation of the total protein levels in this assay.

A



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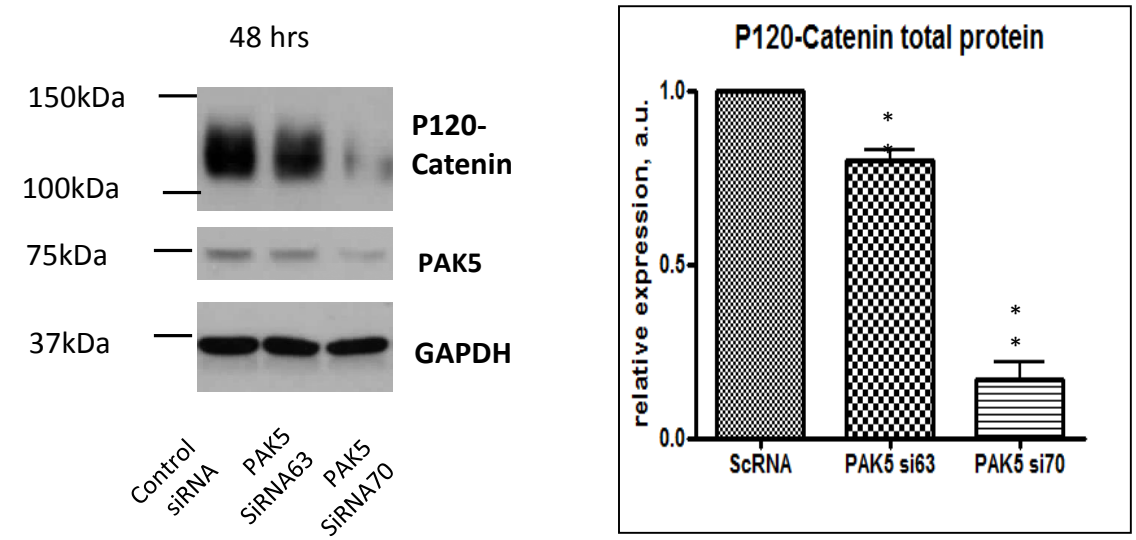


Figure 4-15 **: The effects of PAK5 siRNA knockdown** at 48 hours on the total protein levels of A) E-Cadherin, and B) P120-Catenin in RT4 cells. Western blots for total protein levels were quantified for relative expression normalised to GAPDH. Relative expressions quantified represent the mean of 3 independent experiments. Statistical significance compared with Scrambled SiRNA (ScRNA) was calculated using Student's *t*-test; **, P, 0.05

4.3 Discussion

The protein expression of PAK5 in bladder cancer cell lines indicated that PAK5 may be downregulated as the urothelial cancer cells progress from well differentiated epithelial morphology to become more mesenchymal. I had investigated whether the down-regulation of PAK5 can also be detected at mRNA transcription level in patient derived tissue samples, as well as the bladder cancer cell lines in the panel. The results for PAK5 mRNA expression from patient specimen cohort provided by my collaborators in Cardiff indicated that PAK5 levels were reduced in tumour tissues compared to normal bladder urothelium. However, the results for Cardiff cohort were not statistically significant, possibly due to the sample size and outliers.

The hypothesis for the downregulation of PAK5 in bladder tumours compared to normal bladder tissues was further supported by the profile for PAK5 mRNA expression available on Gene Expression Omnibus ((GEO: GPL96, 213990_s_at (ID_REF), GDS1479, 57144) associated publication (Dyrskjot, Kruhoffer et al. 2004)), where statistically significant downregulation of PAK5 mRNA was detected in bladder tumours, compared to normal bladder tissues.

Despite the downregulation of PAK5 mRNA in bladder tumours compared to normal urothelium, differences or downregulation of PAK5 as the tumours progress to higher grades and stages were not detected. This indicated that PAK5 mRNA expression in bladder cancer may not comprehensively translate to its protein expression. The mRNA expression of PAK5 in bladder cancer cell lines was therefore assayed, which showed a different expression profile, compared to its distinct protein expression in these cell lines. The difference in the profile for mRNA and protein expression, albeit unexpected, may be accounted for by translational or post translational regulation of PAK5. The mechanism for this discrepancy between the transcription and translation of PAK5, or any other isoforms of PAKs has not been characterised in detail. However, one specific group of molecules which had recently gained attention that can affect the translation of PAK mRNAs to PAK proteins in bladder cancer are microRNAs.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs of 19 to 25 nucleotides in length that regulate gene expression, and it is estimated that these molecules regulate up to 30% of human genes by targeting mRNAs for cleavage or translational repression (Bartel 2004, Garzon, Calin et al. 2009, Spizzo, Nicoloso et al. 2009). A number of microRNAs had been identified to target PAKs. PAK1 mRNA has been reported to be a target of miR-7 and also Let-7 (Reddy,

Ohshiro et al. 2008, Hu, Guo et al. 2013). PAK4, a member of group 2 PAKs, has also been shown to be targeted by microRNAs such as miR-224 and miR-145 in cancer studies (Wang, Zhang et al. 2012, Zhang, Takahashi et al. 2013). In urothelial cancer studies, deregulation miRNA-145, a potential tumour suppressor, has been characterised, where low expression of miR-145 has been associated with cancer progression, and a marker of poor prognosis (Ostenfeld, Bramsen et al. 2010, Villadsen, Bramsen et al. 2012). Conversely, in addition to downregulation of tumour suppressor miRNAs,, upregulation of oncogenic miRNAs such as miR-183, miR-96, miR-96, miR17-5p and miR-20a have also been described in urothelial cancer (Yoshino, Seki et al. 2013). To date, microRNAs targeting human PAK5 have not yet been identified or characterised, and the discrepancy in the mRNA and protein expression of PAK5 in these bladder cancer cell lines may provide useful resources for such study.

As the protein expression of PAK5 in bladder cancer cell lines were closely related to epithelial differentiation and morphology, I focussed on characterisation of endogenous PAK5 expression in well differentiated urothelial cancer cell line RT4. Indirect immunofluorescence and confocal microscopy have identified distinct distribution of PAK5 at the cell membrane associated with cell-cell adherens junction and colocalisation with E-cadherin. This is a novel finding, as endogenous PAK5 had only been localised to the cell membranes (and filopodia) in differentiated LAN neuroblastoma cells (Matenia, Griesshaber et al. 2005). A study by Wu et al on PAK5 localisation had demonstrated that PAK5 was spatially regulated by its interaction with Rho GTPases at its CRIB domain, where co-expression of PAK5 with Cdc42 directed PAK5 to the cell membrane, but not the expression of PAK5 alone or co-expression with another Rho GTPase RhoD (Wu and Frost 2006).

The roles or functions for PAK5 subcellular localisation to the cell membrane have not been well characterised. PAK5 have been shown to interact, and directly phosphorylate p120-Catenin on serine 288 (Wong, Reynolds et al. 2010). P120-catenin is one of the constitutional proteins of the Cadherin-Catenin adherens junction complex, and the study co-localised PAK5 with P120-Catenin (phosphor s288) in discrete punctate distribution within the cytoplasm of mouse fibroblast NIH-3T3 cells. The study however did not characterise the functional role of this interaction, nor linked PAK5 to cadherin-catenin complex, as the cells used did not express E-cadherin, or form characteristic epithelial cell-cell adhesion.

Downregulation of PAK5 in RT4 cells by siRNA technology was performed in my study to further understand the role of PAK5 in urothelial tumours. Two siRNA oligonucleotides were deployed to downregulate PAK5 protein expression. At 48 hours following siRNA transfection, maximal effects of the assay was detected for the PAK5 protein expression, associated with changes in RT4 cell morphology, stability of cell-cell adherens junctions, and downregulation of total protein levels of E-cadherin and P120-catenin.

In order to present the findings on the morphological changes and the state of cell-cell adhesion in RT4 following transient PAK5 siRNA knockdown, I proposed an alternative quantitative approach, based on the model of cell-cell adhesion formation and maturation in 3 different stages (Adams, Chen et al. 1998, Vasioukhin, Bauer et al. 2000, Vasioukhin and Fuchs 2001). This quantitative method was also a modification to junctional index quantification previously described (Cain, Vanhaesebroeck et al. 2010). Junctional index, which was calculated using the formula ([junctional area/total area] × 100)/cell number, may not accurately represent the morphological changes due to a number of reasons. Firstly, following PAK5 siRNA knockdown, the total cell area of RT4 cells decreased with concurrent reduction in the area of cell-cell contact. The decrease in both parameters will directly increase the sum of the junctional index calculated, where the loss of cell-cell contact may be underestimated. Secondly, junctional index calculation was modelled on (endothelial) cells in a monolayer, whereas my assay, RT4 cells were seeded at low density to achieve small epithelial colonies of cells, in order to detect dissociation of cell-cell adhesion. Thirdly, increase in junctional area parameter may also misrepresent the stability of the adherens junction, as the E-cadherin in membranous adhesion plaques in mature epithelial junctions are redistributed into larger aggregates of punctae in less mature (stages 1 and 2) epithelial junctions, and may increase in the area for cell-cell adhesion (Adams, Chen et al. 1998).

The morphological changes and dissociation of cell-cell adherens junctions in RT4 are likely to be the direct effects of E-cadherin and P120-Catenin downregulation in this assay. The mechanism for which PAK5 siRNA knockdown affected the total protein levels of E-Cadherin and P120-catenin was unclear. What is already known, however, is that p120-catenin regulates cadherin turnover and is required for cadherin stability. It could be speculated that the reduction in the total E-cadherin protein levels to be the results of p120 downregulation, resulting in rapid degradation of E-cadherin by lysosomal destruction (Fujita, Krause et al. 2002, Palacios, Tushir et al. 2005). Serine threonine phosphorylation of P120-catenin by PAK5, and the loss of this

interaction following PAK5 downregulation may play a significant role in this process. In contrast to growth-factor stimulated tyrosine phosphorylation of P120-catenin, the role of its serine/threonine phosphorylation had not been well understood. Studies had demonstrated that serine/threonine residues of P120-Catenin were constitutively phosphorylated in many cell lines with epithelial differentiation such as MDCK, MCF-7, HCT-116 and A431; and p120 serine/threonine phosphorylation positively correlated with the E-cadherin levels in the cells (Xia, Mariner et al. 2003, Xia, Carnahan et al. 2006). In my siRNA knockdown assay, I was unable to clarify how the loss of PAK5 affected the serine/threonine phosphorylation of p120, as the total protein level was already reduced, possibly through degradation.

A recently published research on the PAK5 in hepatocellular carcinoma had demonstrated that PAK5 silencing also downregulated the total pool of β-catenin protein expression (Li, Yao et al. 2013). In epithelial adherens junctions, β-catenin is one of the core protein components; through its ability to bind to α-catenin and link E-Cadherin to the actin cytoskeleton. However, β-catenin leads a dual 'life' that it can also act as a transcriptional co-factor when stimulated by the Wnt signal transduction pathway, implicated in cancer progression and metastasis(MacDonald, Tamai et al. 2009). The pool of β-catenin associated with Wnt-signalling pathway had thus far thought to be separate and functionally distinct from adherens-junctions related β-catenin, and the functions have been intensely studied, sometimes in mutually exclusive settings. In a review, (Nelson and Nusse 2004) the authors had elegantly summarised this issue by asking a direct question: "Can the cadherin bound pool of β-catenin be released and made available for signalling?". The answer to this question is emerging, where it had been demonstrated that cadherin-bound β-catenin feeds into the Wnt pathway and nuclear translocation upon adherens junctions dissociation, which provided the evidence for intersection between the two β-catenin pools (Kam and Quaranta 2009). It is therefore unclear whether the downregulation of PAK5 which resulted in β-catenin loss, as reported in hepatocellular carcinoma, could also affect adherens-junctions formation.

Following PAK5 siRNA knockdown, RT4 cells also demonstrated morphological changes associated with 'cell-rounding', as demonstrated by cell-shape analysis which showed decreased cell area, and increased circularity and aspect ratio (figure 4.11). The circular morphology associated with loss of cell-cell adhesion due to E-cadherin inactivation in RT4 cells had previously been described in a study when RT4 cells were cultured in the presence of anti-E-

Cadherin function-blocking antibody (Bryan, Atherfold et al. 2008). In addition to loss of E-cadherin adhesive functions, loss of p120-catenin, as seen in PAK5 knockdown can also result in cell-rounding through deregulation of Rho-GTPases. P120-catenin is a potent regulator of the Rho family of small GTPases, which regulate the cytoskeletal dynamics (Yanagisawa, Huveldt et al. 2008) (Anastasiadis 2007). Specifically, p120-catenin inhibits RhoA; and decrease in p120 levels in PAK5-siRNA RT4 cells could be speculated to increase RhoA activity. Increase in RhoA signalling promotes actin-contractility, cell rounding, and rounded-cell motility (Sahai and Marshall 2003), and may be implicated in the morphological changes observed in PAK5-siRNA RT4 cells.

In the next chapter, I aimed to further characterise the interaction of PAK5 with Cadherin-Catenin protein complex, and its role in the maintenance of at the cell-cell adherens junctions.

Chapter 5 : Interactions between PAK5 and E-cadherin

5.1 Introduction

I have found that PAK5 protein expression levels were higher in bladder cancer cell lines with differentiated epithelial/colony-forming morphology compared to poorly differentiated cell lines. In RT4 and RT112 cells, PAK5 colocalised with E-cadherin at the cell-cell adherens junctions in basal growth conditions. SiRNA silencing of PAK5 in RT4 cells inhibited the maturation of cell-cell adherens junctions, and loss of PAK5 expression was associated with downregulation of the core junctional proteins; E-Cadherin and p120-Catenin.

The presence of adherens junctions (AJs) is a defining feature of all epithelial sheets (Fristrom 1988, Baum and Georgiou 2011). The prototypical protein that forms the transmembrane core of adherens junctions is E-cadherin (figure 5.1). The understanding of E-Cadherin structure and domains is crucial to further characterise the interaction between PAK5 and E-Cadherin. E-Cadherin has an ectodomain composed of five extracellular cadherin (EC) repeats, a single transmembrane region and a cytoplasmic domain (Shapiro and Weis 2009). Its extracellular domain is responsible for homotypic, calcium dependent interactions with E-Cadherins on the surface of adjacent cells.

The cytoplasmic domain of E-Cadherin is 150 amino acids long, and is the most highly conserved domain in the Cadherin family of proteins (Nollet, Kools et al. 2000). It interacts with p120-Catenin and β–catenin through its juxtamembrane and catenin-binding domains respectively. β–catenin provides linkage between adherens junctions and the actin cytoskeleton through interactions involving α–catenin (Vasioukhin and Fuchs 2001, Nelson 2008).

Newly synthesized E-Cadherin associates with β–catenin in the endoplasmic reticulum and these associated proteins are transported together to the cell membrane in the formation of cell-cell adherens junctions (Hinck, Nathke et al. 1994). The Catenin-binding region of Cadherins contains a sequence motif that is recognised by ubiquitin ligases, and disengagement of E-Cadherin/β–catenin binding exposes this sequence which leads to proteosomal degradation of E-Cadherin (Huber, Stewart et al. 2001, Huber and Weis 2001).

P120-catenin also stabilises E-cadherin at the cell-cell adherens junctions (Ireton, Davis et al. 2002). P120 acts at the cell adherens junctions to control cadherin turnover, and p120 siRNA knockdown resulted in dose dependent elimination of E-cadherin and loss of cell-cell adhesion (Davis, Ireton et al. 2003). Downregulation of p120-catenin occurs frequently in cancer tissues, but paradoxically is uncommon in established cancer cell lines (Ireton, Davis et al. 2002, Stairs, Bayne et al. 2011). In the absence of E-Cadherin, p120 is stable in the cells, but had been described to be 'stranded' in the cytoplasm (Thoreson, Anastasiadis et al. 2000). PAK5 has previously been reported to interact with P120 *in vivo* using mouse brain extract, and *in vitro* pull-down assays in N1E115 and HeLa cells, but this has not been tested in cells with a conserved epithelial morphology(Wong, Reynolds et al. 2010).

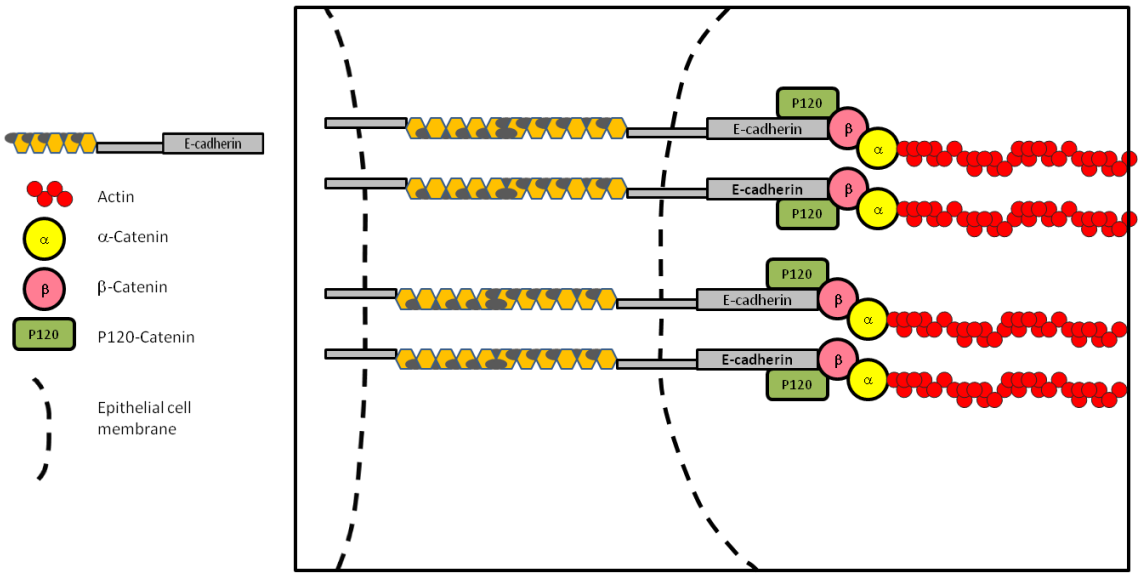


Figure 5-1: Model for E-Cadherin-Catenin protein complex at epithelial cell-cell adherens junctions

In this chapter, I report the findings of further investigations on the interactions of PAK5 with the cadherin-catenin complex, utilising bladder cancer cell lines expressing endogenous PAK5, as well as over expressed PAK5-fusion proteins.

5.2 Results

5.2.1 PAK5 co-immunoprecipitated with E-cadherin, p120-catenin and β-catenin in RT4 cells

I had discovered that in RT4 cells, PAK5 colocalised with E-Cadherin in adherens junctions, and siRNA knockdown of PAK5 resulted in reduced protein levels of E-Cadherin and p120-Catenin, associated with cell-cell dissociation from epithelial colonies. I therefore hypothesised that in RT4 cells, endogenous PAK5 may interact with proteins in the Cadherin-Catenin adherens junction complex.

A common and rigorous assay that can be performed to demonstrate the protein-protein interaction is co-immunoprecipitation (Berggard, Linse et al. 2007). High basal level of protein expression for PAK5 and E-Cadherin in RT4 cells had presented a potential cell-line model to investigate the interaction between PAK5 and the adherens-junctions proteins, where the proteins were in their native conformation. To test this hypothesis, I performed co-immunoprecipitation (Co-IP) assays and Western blots for endogenous PAK5 and adherens-junctions proteins using the whole cell lysates of RT4 cells cultured in basal growth conditions. Precipitates from RT4 lysates incubated with control (anti-HA) antibody was used as negative control.

I first investigated the interaction between PAK5 and E-cadherin. Using the in-house PAK5 antibody. When PAK5 was used as the bait, E-Cadherin was co-immunoprecipitated (figure 5.2, lanes 2a and b). In parallel, this interaction was also validated by using E-Cadherin as the bait, PAK5 was co-immunoprecipitated with E-Cadherin (figure 5.2, lanes 3a and b).

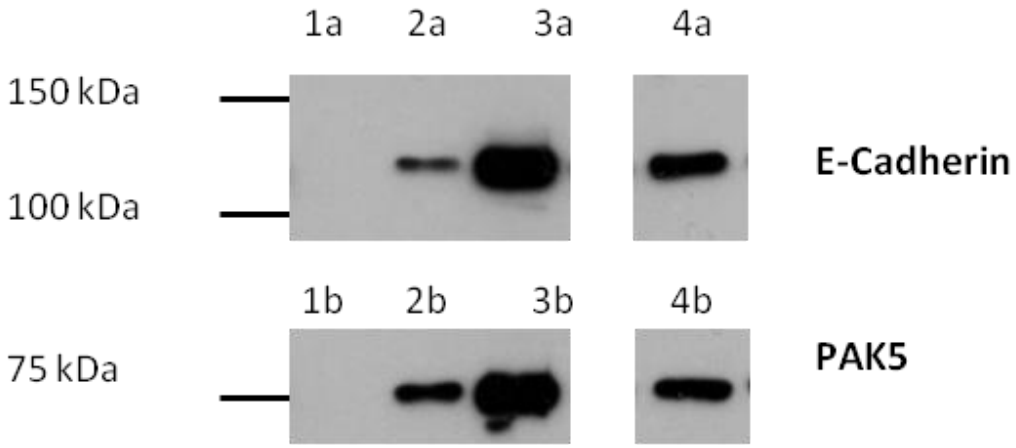
The co-immunoprecipitation of PAK5 with E-cadherin in RT4 cells had provided further evidence that PAK5 may be involved in the regulation of epithelial adherens-junctions. Thus, I proceeded to test whether PAK5 also interacted with other core proteins in the adherens-junctions complex; p120-Catenin, β-Catenin and α-Catenin.

The interaction between PAK5 and p120-Catenin had previously been described in neuronal and HeLa cells (Wong, Reynolds et al. 2010), but not in epithelial cells with established cell-cell-adhesions. Initially, I tested PAK5 immunoprecipitates for co-immunoprecipitation of p120-Catenin. I was unable to detect the presence of P120-Catenin in the dissociated protein lysates

(figure 5.3, lanes 2a-b). However, in the immunoprecipitates of P120-Catenin, PAK5 was detected on the Western blots (figure 5.3, lanes 3a-b).

Having confirmed that PAK5 co-immunoprecipitated with E-Cadherin and p120-Catenin under certain conditions, I proceeded to investigate whether PAK5 was also associated with β- and α-Catenins; 2 other well characterised components of the adherens-junctions complex. In these experiments, E-Cadherin immunoprecipitates were nominated as positive controls. I was able to detect co-immunoprecipitation of PAK5 with β-catenin (figure 5.4, lane 2a). However, co-immunoprecipitation of PAK5 with α-catenin was not detected in the precipitates of PAK5 antibody (figure 5.4, lane 2b). Both β- and α-Catenins were detected in the E-Cadherin immunoprecipitates (figure 5.4, lanes 3a-b).

These results of the co-immunoprecipitation assays indicate that there is an interaction between PAK5 and the adherens- junction complex. The most conclusive evidence for the interaction was seen in the co-immunoprecipitation assays for PAK5 and E-Cadherin (figure 5.2), which could be reversed.



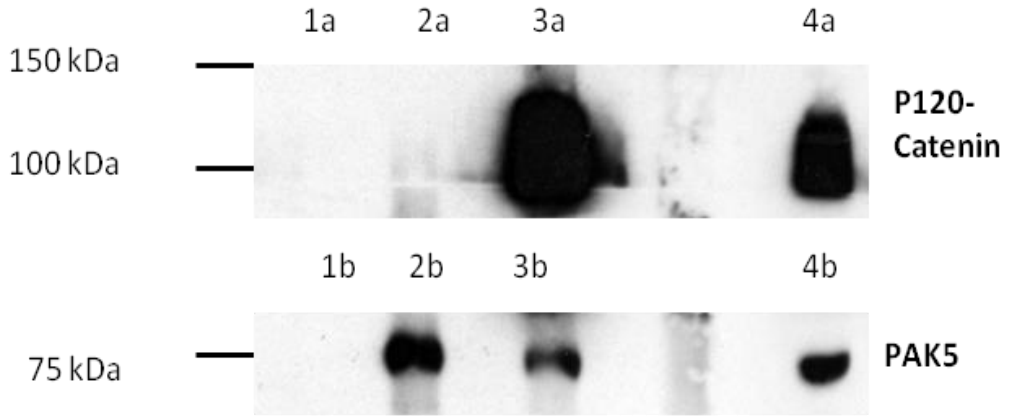
Lanes 1a & b: Immunoprecipitates of HA antibody (negative control)

Lanes 2a & b: Immunoprecipitates of PAK5 antibody

Lanes 3a & b: Immunoprecipitates of E-Cadherin (HECD) antibody

Lanes 4a & b: Whole cell lysates of RT4

Figure 5-2 : **Co-immunoprecipitation of endogenous PAK5 with E-Cadherin in RT4 cells**. (A) RT4 whole cell lysates were incubated with PAK5 or E-Cadherin antibody, followed by Sepharose™ beads. Precipitated proteins were dissociated and analysed by western blots with antibody against PAK5 and E-Cadherin. Images are representative of 3 independent experiments.



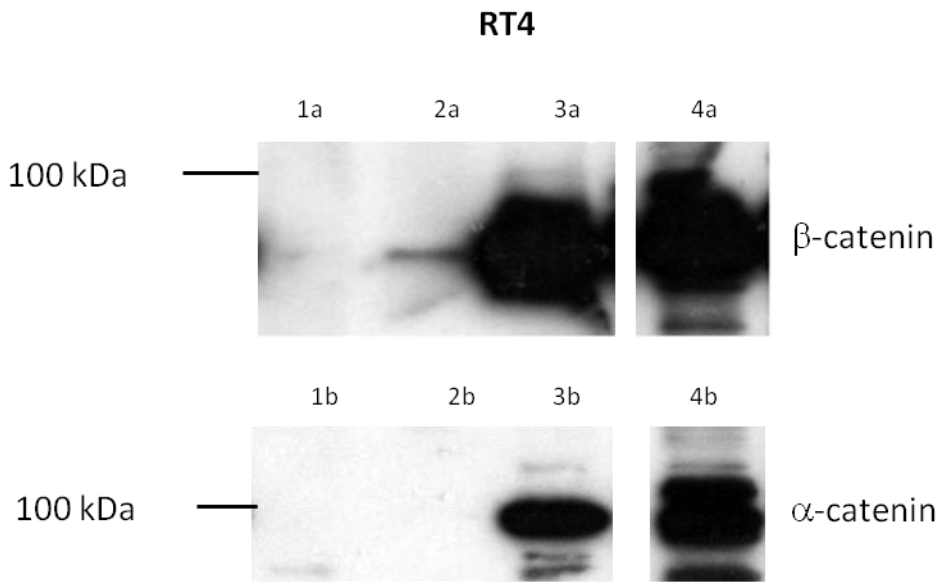
Lanes 1a & b: Immunoprecipitates of HA antibody (negative control)

Lanes 2a & b: Immunoprecipitates of PAK5 antibody

Lanes 3a & b: Immunoprecipitates of p120-Catenin antibody

Lanes 4a & b: Whole cell lysates of RT4

Figure 5-3 : **Co-immunoprecipitation assays for endogenous PAK5 with p120-Catenin in RT4 cells**. RT4 whole cell lysates were incubated with PAK5 or p120-Catenin antibodies, followed by Sepharose beads. Precipitated proteins were dissociated and analysed by western blots with antibody against PAK5 and p120-catenin. Images are representative of 3 independent experiments.



Lanes 1a & b: Immunoprecipitates of HA antibody (negative control)

Lanes 2a & b: Immunoprecipitates of PAK5 antibody

Lanes 3a & b: Immunoprecipitates of E-Cadherin (HECD) antibody

Lanes 4a & b: Whole cell lysates of RT4

Figure 5-4 : **Co-immunoprecipitation assays for endogenous PAK5 with α- and β-Catenin in RT4 cells.** (A) RT4 whole cell lysates were incubated with PAK5 or E-Cadherin antibodies, followed by Sepharose™ beads. Precipitated proteins were dissociated and analysed by western blots with antibody against β- and α-catenins. Images are representative of 3 independent experiments.

5.2.2 PAK5 co-immunoprecipitates with E-cadherin and β-catenin in RT112 cells

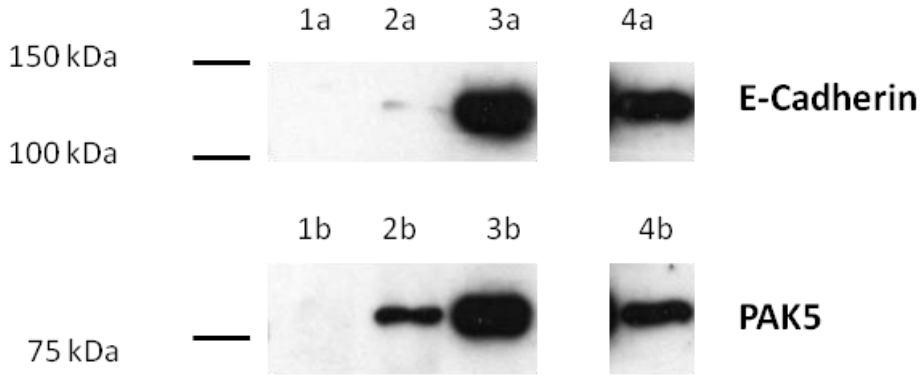
I had demonstrated that the endogenous protein levels of E-cadherin and PAK5 detectable at lower intensity in RT112 compared to RT4 cells (figures 3.5 and 3.15). The cell-cell adherens junctions in RT112 were also less mature compared to RT4 (figure 3.6). As I had previously hypothesised that PAK5 may have a supportive role to stabilise the adherens junctions, I investigated whether PAK5 had a different interaction profile with the adherens-junction proteins in RT112 compared to RT4 cells.

Similar to the experiments described in section 5.2.2, the whole cell lysates of RT112 cells were incubated the specified immunoprecipitation antibodies and the precipitated proteins were analysed by Western blotting. Precipitates from RT112 lysates incubated with anti-HA antibody were used as negative control.

The co-immunoprecipitation between PAK5 and E-cadherin in RT112 cells was first assayed by using the in-house PAK5 antibody. When PAK5 was used as the bait, I was able to co-immunoprecipitate both PAK5 and E-Cadherin, and this was demonstrated when the precipitated protein complex were dissociated and assayed by Western blotting (figure 5.5, lanes 2a, and b). This interaction was further validated using E-Cadherin as the bait, in which the whole cell lysates of RT112 was incubated with E-Cadherin specific (HECD) antibody. The interaction between PAK5 and E-cadherin was also detected in the co-immunoprecipitation assay using E-Cadherin antibody (figure 5.5, lanes 3a, and b). I had also noted that the E-Cadherin antibody was able to co-immunoprecipitate PAK5 and E-Cadherin more efficiently than PAK5 antibody in these experiments.

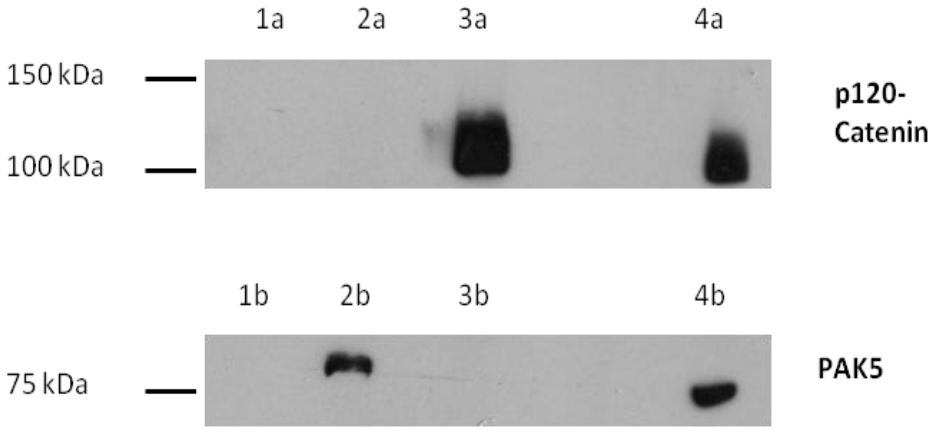
The interaction between PAK5 and P120 catenin was also investigated by co-immunoprecipitation assays in RT112 cells by using PAK5 or E-Cadherin antibody. Using PAK5 antibody, I was able to detect the immunoprecipitation of PAK5 (the bait), but not p120-Catenin (figure 5.6, lanes 2a, b). Co-immunoprecipitation of PAK5 with p120-Catenin was also not detected when the lysates of RT112 cells were incubated with p120-Catenin antibody (figure 5.6, lanes 3a, and b).

The interactions demonstrated by the co-immunoprecipitation between PAK5 with β- and α-catenins in RT112 cells were similar to the interactions previously observed in RT4 cells. In the Western blots for the precipitates of PAK5 antibody form RT112 cells, I was able to detect co-immunoprecipitation of PAK5 with β-catenin (figure 5.7, lane 2a). Co-immunoprecipitation of PAK5 with α-catenin was not detected in the precipitates of PAK5 antibody (figure 5.7, lane 2b). In these experiments, precipitates of RT112 lysates incubated with E-Cadherin antibody were nominated as positive controls. Both β- and α-catenins were detected in the immunoprecipitates of E-Cadherin antibody (figure 5.7, lanes 3a-b).



Lanes 1a & b:	Immunoprecipitates of HA antibody (negative control)
Lanes 2a & b:	Immunoprecipitates of PAK5 antibody
Lanes 3a & b:	Immunoprecipitates of E-Cadherin antibody
Lanes 4a & b:	Whole cell lysates of RT112

Figure 5-5 : **Co-immunoprecipitation of endogenous PAK5 with E-Cadherin RT112 cells.** RT112 whole cell lysates were incubated with PAK5 or E-Cadherin antibodies, followed by Protein A Sepharose beads. Precipitated proteins were analysed by western blots with antibody against PAK5 and E-Cadherin. Images are representative of 3 independent experiments.



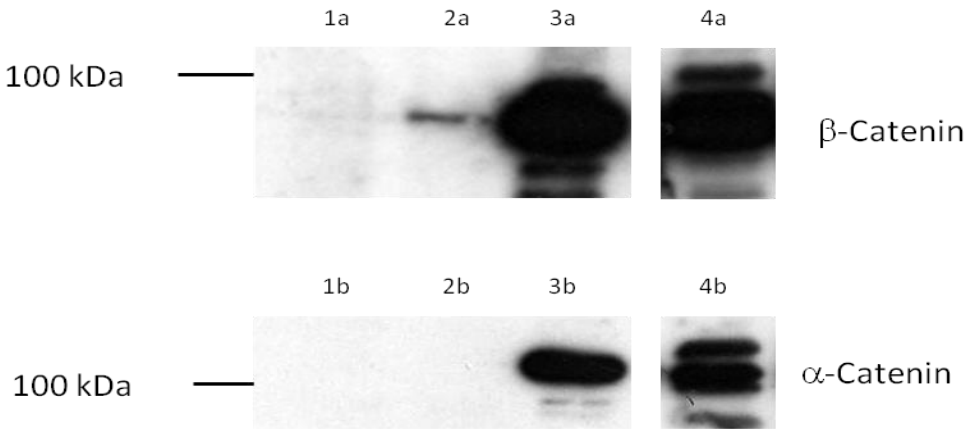
Lanes 1a & b: Immunoprecipitates of HA antibody (negative control)

Lanes 2a & b: Immunoprecipitates of PAK5 antibody

Lanes 3a & b: Immunoprecipitates of p120-Catenin antibody

Lanes 4a & b: Whole cell lysates of RT112

Figure 5-6 **: Co-immunoprecipitation assays for endogenous PAK5 with α- and β-Catenin in RT112 cells.** RT112 whole cell lysates were incubated with PAK5 or p120-Catenin antibodies, followed by Sepharose™ beads. Precipitated proteins were analysed by Western blots with antibody against β- and α-catenins. Images are representative of 3 independent experiments.



Lanes 1a & b: Immunoprecipitates of HA antibody (negative control)

Lanes 2a & b: Immunoprecipitates of PAK5 antibody

Lanes 3a & b: Immunoprecipitates of E-Cadherin antibody

Lanes 4a & b: Whole cell lysates of RT112

Figure 5-7 **: Co-immunoprecipitation assays for endogenous PAK5 with α- and β-Catenin in RT112 cells**. (A) RT112 whole cell lysates were incubated with PAK5 or E-Cadherin antibodies, followed by Sepharose™ beads. Precipitated proteins were analysed by western blots with antibody against β- and α-catenins. Images are representative of 3 independent experiments.

5.2.3 N-terminal (regulatory domain) PAK5 regulates the interaction between PAK5 and E-Cadherin

The strong interaction between PAK5 and E-cadherin was a novel finding, and required further characterisation. PAK5, similar to other isoforms of PAKs contain an N-terminal regulatory domain and a highly conserved C-terminal catalytic kinase domain(Eswaran, Soundararajan et al. 2008). Located within the N-terminus of PAK5 are regulatory sequences which include the Cdc42/Rac interactive binding (CRIB) domain, the auto-inhibitory domain (AID), nuclear localisation/export sequences, mitochondria targeting sequences (Cotteret and Chernoff 2006). The Cdc42/Rac interactive binding (CRIB) domain, positioned the N-terminal, has also been reported to play a critical role for proper spatial targeting of PAK5, and interactions with specific members of the Rho-family of GTPases targets PAK5 to distinct subcellular locations (Wu and Frost 2006).

Although PAK5 has been shown to phosphorylate its downstream effectors such as RAF-1 and BAD (Cotteret, Jaffer et al. 2003, Cotteret and Chernoff 2006, Wu, Carr et al. 2008), increasing evidence indicates that some aspects of PAK5 regulation of cytoskeletal reorganisation does not require its kinase activity (Matenia, Griesshaber et al. 2005, Wong, Reynolds et al. 2010). A kinase-independent function, which could indicate a scaffolding role for PAK5, as has also been described for other isoforms of PAK (Frost, Khokhlatchev et al. 1998, Higuchi, Onishi et al. 2008).

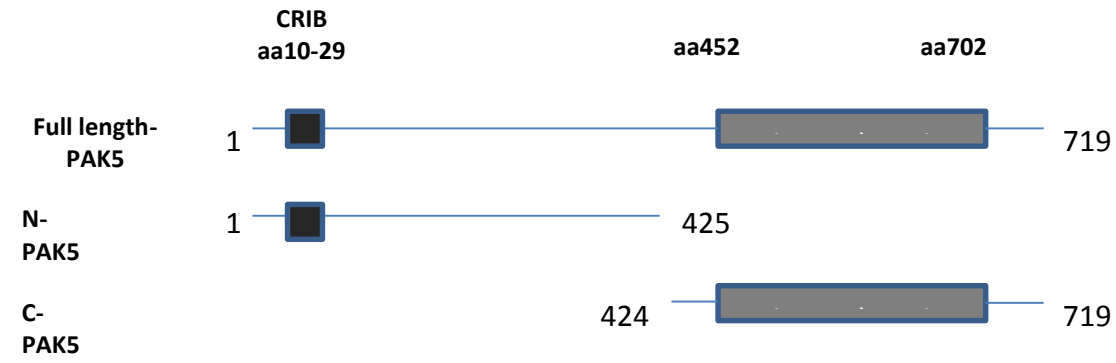
In order to investigate whether the interaction between PAK5 and E-Cadherin was mediated via its N-terminal regulatory domain or it C-terminal kinase domain, I generated PAK5 N-terminal (N-PAK5) and C-terminal (C-PAK5) mutants (figure 5.5A) by polymerase chain reaction (PCR) and Gateway™ technology primers (forward and reverse primers listed in the chapter 2). The C-PAK mutant construct (aa 425-719) included the complete kinase domain of PAK5 (Eswaran, Lee et al. 2007). The cDNA for all the constructs were sequenced to confirm that no mutations had arisen in the DNA sequence during the cloning process.

The PCR products for N-PAK5 and C-PAK5 mutants (figure 5.5B) were cloned into Gateway™ (modified) RFP destination vector, with the RFP-tag positioned at the amino- termini of the corresponding PAK5 protein domains. As the interaction between PAK5 and E-cadherin was detected most robustly in RT4 cells, and in these cells the proteins colocalised at the cell-cell

junctions, I investigated where thePAK5 domain mutants were localised compared to full-length PAK5.

When I overexpressed the RFP-tagged PAK5 constructs in RT4 cells, I observed distinct changes to the distribution of C-PAK5 compared to FL-PAK5 or N-PAK5 (figure 5.5C). FL-PAK5 and N-PAK5 were both expressed in the cytoplasm in vesicular/punctate distributions, and excluded from the nucleus. C-terminal domain of PAK5 however accumulated in the nucleus, with diffuse (non-vesicular) cytoplasmic distribution. The strong nuclear accumulation and diffuse cytoplasmic distribution of endogenous PAK5 had not been observed in RT4 cells in steady state.

A



B

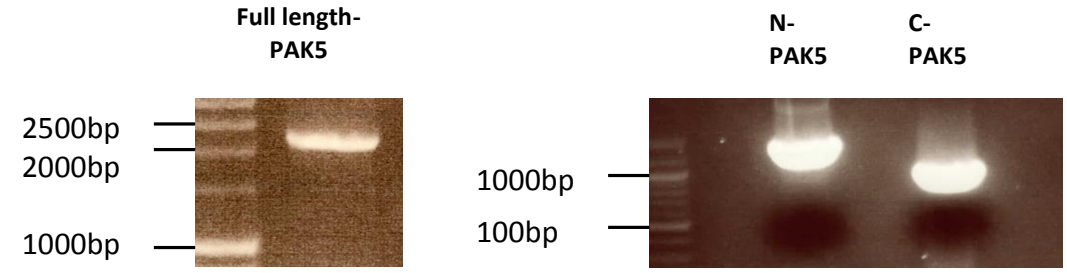


Figure 5-8 : **Generation of PAK5 N-terminal and C-terminal mutants**. A) Schematic representation of full length PAK5 and N- and C-termini PAK5 protein fractions. B) PCR products of full length PAK5, and N- and C-termini mutants cloned into Gateway™ expression vectors.

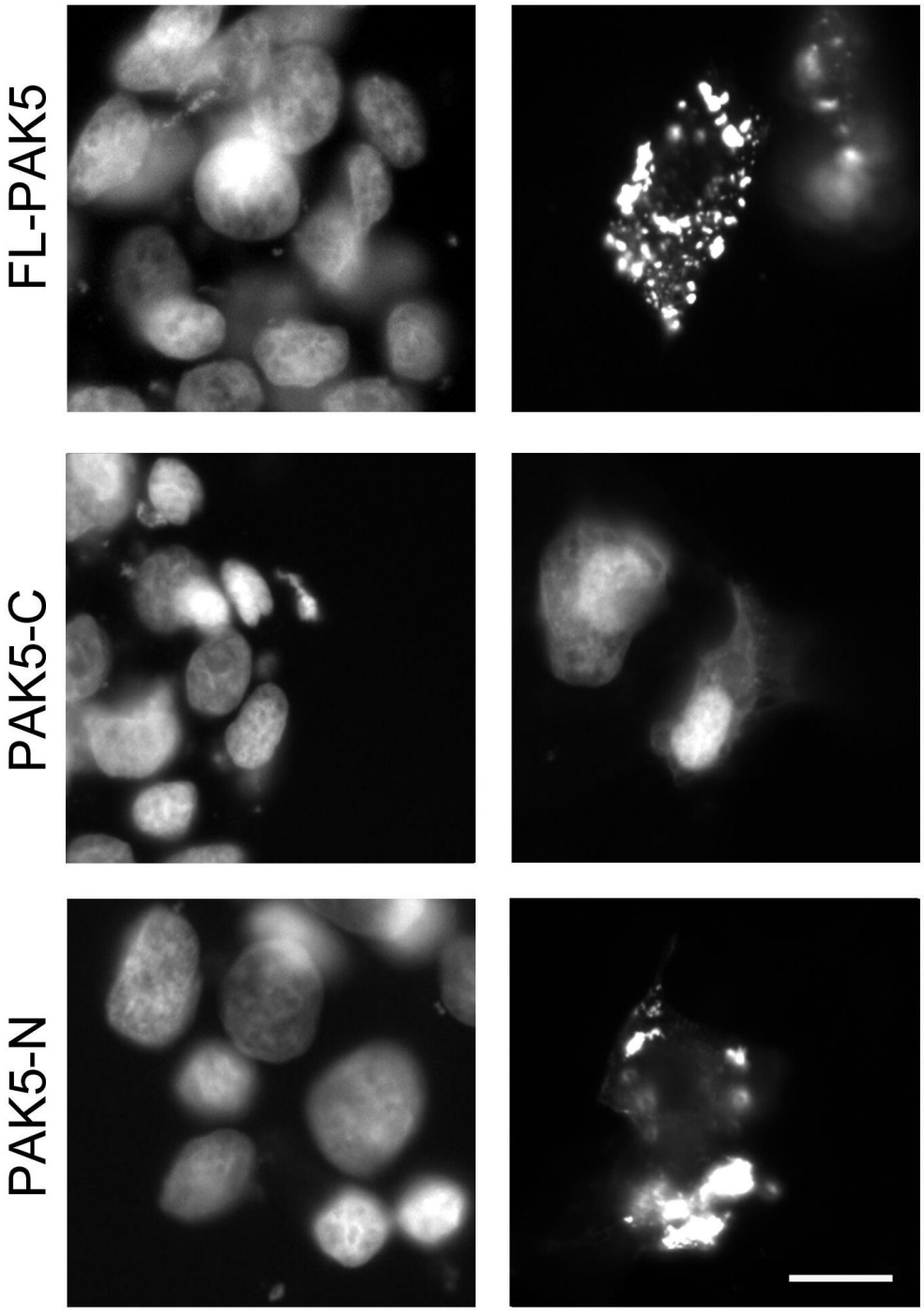


Figure 5-9 : **Fluorescent images of RT4 cells transiently transfected with RFP-tagged full length PAK5, and N- and C-termini mutant. Nuclei were stained with DAPI**, A) RFP-FL-PAK5 was distributed in punctate distribution in RT4 cells. B) N-terminal PAK5 protein was also distributed in distinct punctate. C) C-terminal PAK5 was distributed in RT4 cells in diffuse cytoplasmic distribution and nuclear accumulation. Cell images are representative of 30 transfected cells from 3 independent experiments. Bar=10μm.

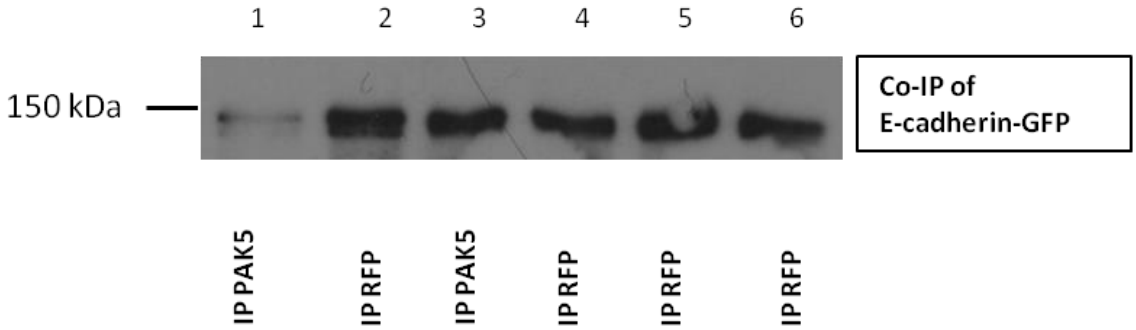
I was unable to transfect RT4 cells efficiently with PAK5 (C- and N-termini) DNA constructs to test my hypothesis and study the protein-protein interaction by pull-down assay. Therefore, as an alternative, HEK293 cell line was used for over expression of PAK5 (C- and N-termini) and E-cadherin due to its high transfection efficiency. HEK293 cells overexpressing E-Cadherin-GFP were co-transfected with FL-PAK5, N-PAK5 or C-PAK5 mutant cDNA constructs. HEK293 cells over-expressing E-Cadherin GFP alone were used as negative control. PAK5 specific antibody previously used in the endogenous PAK5 pull-down assays could only be utilised to immunoprecipitate FL-PAK5 and N-PAK5, but not C-PAK5 as the antibody was designed to interact with the epitope (aa 146-160) within the N-terminal of PAK5.

The first set of pulldown assays were performed by using RFP-tagged FL-, N-, and C-PAK5 constructs, and RFP-antibody was used for the immunoprecipitation assay. The RFP-PAK5 precipitated products were probed for co-immunoprecipitation of E-Cadherin-GFP (figure 5.6A). To test the validity of the interactions observed in the co-immunoprecipitation assay using RFP antibody, I included PAK5 antibody to IP the negative (E-cadherin –GFP only) and positive (co-transfection with E-Cadherin GFP and RFP-FL-PAK5) control lysates. Immunoprecipitation using PAK5 antibody co-precipitated E-cadherin-GFP in the lysates of HEK293 cell overexpressing E-Cadherin GFP and RFP-FL-PAK5, but not E-Cadherin GFP alone (figure 5.5B). Immunoprecipitation using RFP antibody however, also detected direct binding to E-Cadherin GFP, independent of RFP-PAK5 expression in HEK293 cells.

I therefore proceeded to generate PAK5-mutants and FL-PAK5 cloned in Gateway™ HA-expression vector to reduce the non-specific cross-reactivity in the co-immunoprecipitation assay. Using HA-tagged PAK5 constructs, I was able to demonstrate that E-Cadherin overexpressed in HEK293 cells interacted most avidly with FL-PAK5 (figure 5.5B). Interaction between the E-Cadherin and N-PAK5 protein was detected in the pulldown assay, but not between E-cadherin and C-PAK5.

Based on these results, I concluded that the N-terminal regulatory domain of PAK5 had a significant role in the regulation of PAK5 interaction with E-Cadherin. The co-immunoprecipitation of N-PAK with E-Cadherin may indicate a scaffolding role for PAK5 in the interaction.

A



Lane 1: Negative control: Over-expression of E-Cadherin-GFP only
Lane 2: Negative control: Over-expression of E-Cadherin-GFP only
Lane 3: Positive control: Over-expression of E-Cadherin-GFP and RFP-FL-PAK5
Lane 4: Positive control: Over-expression of E-Cadherin-GFP and RFP-FL-PAK5
Lane 5: Over-expression of E-Cadherin-GFP and RFP-N-PAK5
Lane 6: Over-expression of E-Cadherin-GFP and RFP-C-PAK5

B

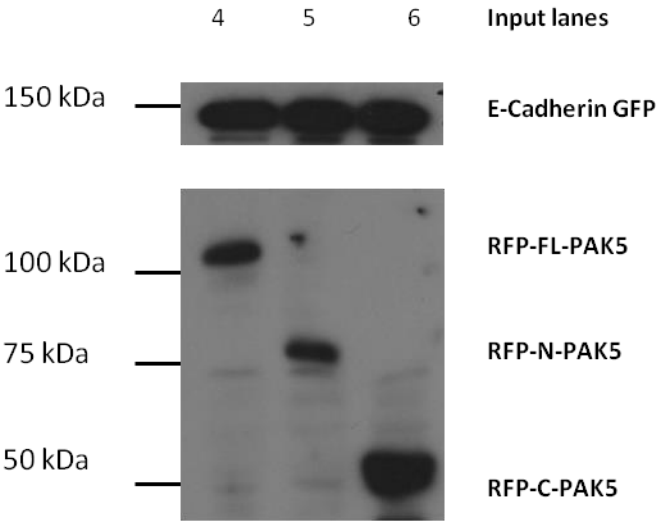
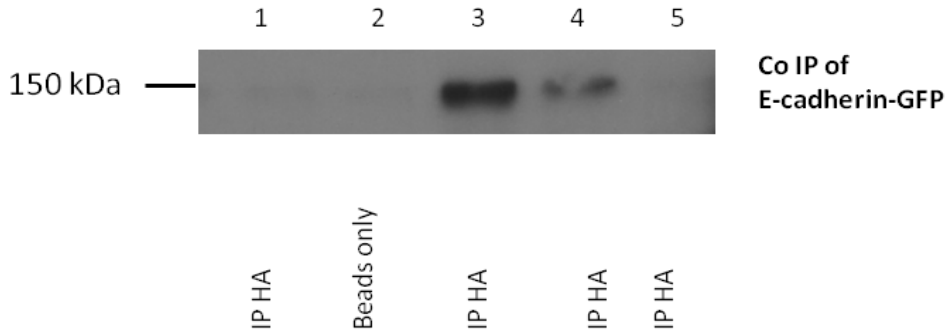


Figure 5-10 **: Interactions between E-Cadherin-GFP and RFP-tagged Full-length PAK5, and N- and C-termini PAK5 fractions overexpressed in HEK293 cells**. A) Whole cell lysates of HEK293 cells transfected with E-Cadherin-GFP with or without RFP-(FL/N/C)-PAK5 were incubated with RFP or PAK5 antibodies as indicated. Precipitated proteins were analysed by Western blots and probed for co-immunoprecipitation of E-Cadherin-GFP. Co-immunoprecipitation of E-Cadherin-GFP and RFP-tagged PAK5 N- or C-termini constructs could not be verified using RFP antibody as direct interaction between RFP antibody with E-Cadherin-GFP protein was observed in the immunoprecipitation of negative control lysates (RFP-PAK5 null cells). PAK5 antibody was used to confirm both positive and negative controls. B) Input lysates for cells over-expressing E-Cadherin-GFP and RFP-(FL/N/C)-PAK5 as indicated by the lane numbers.

A



Lane 1: Negative control: Over-expression of E-Cadherin-GFP only
Lane 2: Negative control: Over-expression of E-Cadherin-GFP and HA-FL-PAK5 (without IP antibody)
Lane 3: Positive control: Over-expression of E-Cadherin-GFP and HA-FL-PAK5
Lane 4: Over-expression of E-Cadherin-GFP and HA-N-PAK5
Lane 5: Over-expression of E-Cadherin-GFP and HA-C-PAK5

B

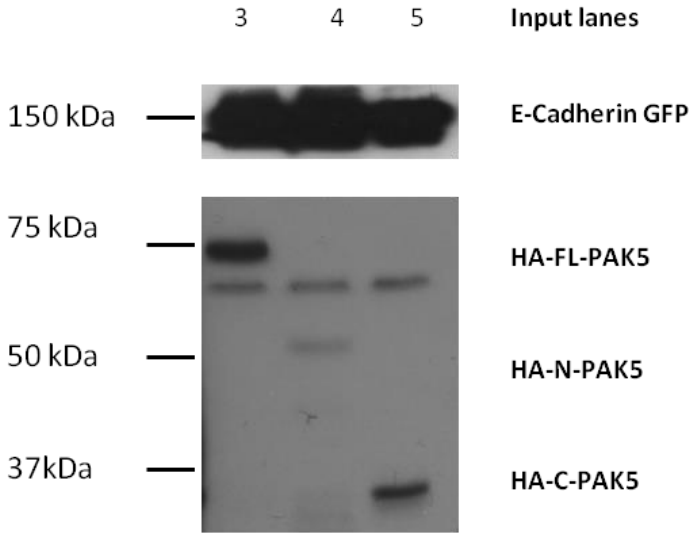


Figure 5-11 : **Interactions between E-Cadherin-GFP and HA-tagged Full-length PAK5, and N- and C-termini PAK5 fractions overexpressed in HEK293 cells**. A) Whole cell lysates of HEK293 cells transfected with E-Cadherin-GFP with or without HA- (FL/N/C)-PAK5 were incubated with HA antibody as indicated. Precipitated proteins were analysed by Western blots and probed for co-immunoprecipitation of E-Cadherin-GFP. B) Input lysates for cells over-expressing E-Cadherin-GFP and HA-(FL/N/C)-PAK5 which correspond with the CO-IP assay as indicated by the lane numbers.

5.3 Discussion

E-Cadherin is a 'classical' member of the Cadherin family of cell surface proteins that plays a fundamental role in the formation of adherens junctions in epithelial tissues (Shibamoto, Hayakawa et al. 1995, Yap, Brieher et al. 1997, Shapiro and Weis 2009). Downregulation or loss of E-Cadherin correlates with increased metastatic potential, associated with the loss of the cellular adhesive properties (Hajra and Fearon 2002, Gumbiner 2005), and E-cadherin loss/switch has also been reported in the tumour progression in bladder cancer (Garcia del Muro, Torregrosa et al. 2000, Popov, Gil-Diez de Medina et al. 2000, Clairotte, Lascombe et al. 2006, Bryan, Atherfold et al. 2008).

The interaction between PAK5 and E-Cadherin in bladder cancer cell lines is a novel discovery of my project, and this chapter attempted to further characterise this interaction. I have applied the in-house PAK5 antibody to co-immunoprecipitate endogenous PAK5 with its interacting partners in 2 bladder cancer cell lines; RT4 and RT112, as these cell lines both have detectable protein expression of PAK5 and E-Cadherin.

The interaction between PAK5 and E-Cadherin was most distinct in RT4 cells. RT4 cells are well differentiated bladder cancer cell lines with conserved epithelial morphology and mature cell-cell junctions in basal growth conditions. In addition, I have also previously demonstrated that siRNA knockdown of PAK5 decreased the area of cell-cell contact (section 4.2.8) and the total protein level of E-Cadherin (section 4.2.10) in RT4 cells. Taken together, I speculated that the protein-protein interaction of PAK5 and E-Cadherin affected the intracellular trafficking and intracellular functions of E-cadherin, by maintaining a steady state of E-Cadherin at the cell-cell adherens junctions.

A well characterised adherens junction protein that has been previously demonstrated to be an interacting partner to PAK5 is p120-Catenin (Wong, Reynolds et al. 2010). In a series of publications, p120-Catenin have been shown to regulate E-Cadherin exocytosis, endocytosis and overall intracellular protein turnover (Chen, Kojima et al. 2003, Davis, Ireton et al. 2003, Xiao, Allison et al. 2003, Xiao, Garner et al. 2005). When p120 is localised to the plasma membrane, the p120 ARM domain associates with the E-Cadherin juxtamembrane region, which results in stabilisation and maturation of adherens junctions (Yanagisawa and Anastasiadis 2006). In RT4

cells, I found that PAK5 also co-immunoprecipitated with p120 catenin (figure 5.3), and it is likely that the interaction between PAK5 and E-Cadherin at the cell adherens junctions involves p120-Catenin. Although p120 has been shown to be a substrate of PAK5 by phosphorylation on serine 288, the role for this interaction remains unclear (Wong, Reynolds et al. 2010). Unlike tyrosine phosphorylation, the role for serine/threonine phosphorylation of p120 has not been extensively characterised. In contrast to the transient tyrosine phosphorylation in response to growth factor stimulation (Mariner, Davis et al. 2004), serine/threonine phosphorylation of p120 appears to be constitutive, with exception to S873 (Xia, Mariner et al. 2003).

In the absence of E-cadherin, p120 is stranded in the cytoplasm where it is mostly (serine/threonine) unphosphorylated, and E-cadherin expression restores both membrane localisation and serine/threonine phosphorylation of p120 (Thoreson, Anastasiadis et al. 2000, Fukumoto, Shintani et al. 2008). Using site- and phosphor-specific antibodies, Xia et al identified that signalling of p120 via serine/threonine phosphorylation requires membrane localisation of p120, and the serine/threonine phosphorylation at individual sites of S268, S288, T310 and T910 was in dependent of E-Cadherin expression (Xia, Carnahan et al. 2006). Wild-type and constitutively active PAK5 have been demonstrated to phosphorylate p120-Catenin on S288, without prominent effect on S268, T310 and T916 (Wong, Reynolds et al. 2010), whereas constitutively active (but not wild-type) PAK4 phosphorylates S288 as well as T310.

Although PAK5 and PAK4 can both phosphorylate p120 on S288, the work by Wong et al had highlighted a number of fundamental differences between these group-2 PAKs in their interaction with p120 (Wong, Reynolds et al. 2010). Firstly, there were no significant difference in the level of phosphorylated S288 in the presence of wild-type or constitutively active PAK5. Secondly, the subcellular co-localisation of PAK5-p120 and PAK4-p120 differed; PAK5 co-localised with p120 (total or pS288) in distinct vesicular/punctate distribution, whereas (constitutively active) PAK4 colocalised with p120 in diffuse cytoplasmic distribution when co-expressed in NIH-3T3 mouse embryonic fibroblast cells. It must also be noted that over-expression of constitutively active PAK4 caused translocation of p120 to the nucleus, whereas the pool of p120 remained in the cytoplasm of NIH-3T3 cells upon PAK5 over-expression. Nuclear p120 may control transcription through direct interaction with Kaiso, which relieves the transcriptional repression of Kaiso target genes (Daniel and Reynolds 1999, Daniel, Spring et al. 2002). Studies have examined the localisation of Kaiso in relation to p120, and have indicated that nuclear Kaiso localisation is decreased when

p120 translocates to the nucleus(Soubry, van Hengel et al. 2005), with relief of repression for Kaiso targets such as WNT11, MMP7 and CyclinD1 with strong links to cancer (Park, Ji et al. 2006, Hong, Park et al. 2010, Musgrove, Caldon et al. 2011).

So how does expression of PAK5 in RT4 cells support the steady state of E-Cadherin at adherens junction? As the work to characterise the interaction is still at its infancy, it can only be speculated that the phosphorylation of S288 on p120 by PAK5 may target p120 to the membrane, therefore stabilising the adherens junction complex, and protecting the protein components of AJ from endocytic pathways. However, one may also speculate that PAK5 may interact directly with E-Cadherin as an adaptor protein to stabilise E-Cadherin at the cell membrane, which in turn maintains the membrane localisation and serine/threonine phosphorylation of p120.

β-catenin also immunoprecipitates with PAK5 in both RT4 and RT112 cells is. As part of adherens junctions, β-catenin binds to the cytoplasmic domain of E-Cadherin and to α-catenin (Shapiro and Weis 2009). In a parallel assay, I was unable to detect protein-protein interaction between PAK5 and α-catenin. In the experiments conducted thus far in my project or any external published studies, it has not yet clear whether the interaction between PAK5 and β-catenin is direct or mediated by E-Cadherin. A number of speculations however can be proposed towards the role of this interaction, as there are evidence that the interaction of E-Cadherin and β-catenin is modulated/strengthened by serine/threonine phosphorylation (Lickert, Bauer et al. 2000, Serres, Filhol et al. 2000, Choi, Huber et al. 2006), and it is possible that in RT4 and RT112 cells, PAK5 may also modulate the stability of cell-cell adherens junctions by serine/threonine phosphorylation. However, a role for PAK5 as a scaffolding protein, which stabilises the adherens junction complex independent of its kinase activity must also be considered, as PAK1, the founding PAK-family member have been shown to serve as a scaffold to facilitate PAK1-Akt pathway at the cell membrane (Higuchi, Onishi et al. 2008). In addition, PAK5 has previously been shown to be involved in the regulation of microtubules and F-actin network, where the binding between PAK5 and MARK2 inhibits the activity of MARK2 towards its target, tau protein independent of phosphorylation/kinase activity (Matenia, Griesshaber et al. 2005).

In order to further interrogate the how PAK5/E-cadherin interact, a number of strategies were considered to determine whether PAK5 interacts with E-Cadherin as a scaffolding (adaptor) protein or by its kinase activity. This includes direct comparison between the affinity of E-Cadherin

binding to wild-type PAK5 (PAK5-WT) or kinase-dead PAK5 (PAK5-NE). However, in the study on group-2 PAKs interaction with p120-catenin, a protein closely associated with E-cadherin at the adherens junctions, no significant differences were demonstrated in the affinity binding or serine/threonine (S288) phosphorylation of p120-catenin between PAK5-WT and PAK5-NE. Therefore, in this project, I have generated PAK5 N-terminal (N-PAK5) and C-terminal (C-PAK5) mutants to investigate whether the interaction between PAK5 and E-Cadherin was mediated via its N-terminal regulatory domain or it C-terminal kinase domain.

When RFP-tagged PAK5 constructs were overexpressed in RT4 cells, I observed distinct changes to the distribution of C-PAK5 compared to FL-PAK5 or N-PAK5 (figure 5.5C). FL-PAK5 and N-PAK5 were both expressed in the cytoplasm in vesicular/punctate distributions, and excluded from the nucleus. C-terminal domain of PAK5 however accumulated in the nucleus, with diffuse (non-vesicular) cytoplasmic distribution. The strong nuclear accumulation and diffuse cytoplasmic distribution of endogenous PAK5 had not been observed in RT4 cells in steady state.

The effect of regulatory domain deletion on the subcellular localisation of C-PAK5 is in agreement with previous reports on the role of PAK5 N-terminal regulatory domain of the subcellular targeting of PAK5 , where a number of N-terminal regions have been identified that regulate the localisation of PAK5; a mitochondrial targeting sequence, a nuclear export sequence, and a nuclear localisation sequence. (Cotteret, Jaffer et al. 2003, Cotteret and Chernoff 2006, Wu and Frost 2006). Deletion of mitochondrial and nuclear targeting sequences causes PAK5 to be retained in the nucleus and suppresses PAK5 cellular activity (Cotteret and Chernoff 2006).

An important sequence located in the N-terminal regulatory domain of PAK5 is the conserved PBD: AID. PAK5 preferentially binds to Cdc42 in the presence of GTP, and the AID motif is essential for this interaction (Pandey, Dan et al. 2002). The interaction between PAK5 and Cdc42 directed substantial fraction of PAK5 to the filopodia within the cell membrane. In addition to Cdc42, PAK5 was also observed to interact with RhoD and RhoH. The interaction with RhoD targets PAK5 to subcellular locations different from those stimulated by Cdc42 (Cotteret, Jaffer et al. 2003, Wu and Frost 2006). Therefore, deletion of the regulatory domain (including PBD: AID) in the C-PAK5 mutant also results in kinase effects of PAK5 independent of Rho-GTPase regulation.

In order to assay the protein-protein interaction between PAK5 and E-Cadherin, HEK-293 cells were used as a cargo for PAK5 over expression to ensure maximal transfection efficiency and protein levels. Although PAK5 constructs were tagged to RFP have previously been used for localisation studies, these constructs were unsuitable for co-expression with E-cadherin –GFP in my set-up for co-immunoprecipitation assays. Experiments to co-immunoprecipitate RFP-PAK5 (FL/N/C) using RFP antibody also detected direct binding of the antibody to E-Cadherin GFP, independent of RFP-PAK5, as seen in the negative control as seen in the lysates HEK293 cells expressing E-Cadherin-GFP alone. I therefore proceeded to generate PAK5-mutants and FL-PAK5 cloned in Gateway™ HA-expression vector to reduce the non-specific cross-reactivity in the co-immunoprecipitation assay. Using HA-tagged PAK5 constructs, I was able to demonstrate that E-Cadherin overexpressed in HEK293 cells interacted most avidly with FL-PAK5 (figure 5.5B). Interaction between the E-Cadherin and N-PAK5 protein was detected in the pull-down assay, but not between E-cadherin and C-PAK5.

The finding of my study which showed that E-cadherin interacts with N-terminal PAK5 highlighted the importance of PAK5 regulatory domain, and suggested a scaffolding role for PAK5 in the modulation of cell-cell adherens junctions.

Chapter 6 : Concluding remarks

Taken together, I speculated that the N-terminal of PAK5 binds onto the cytoplasmic domain of E-cadherin, and this binding was closely associated with p120-Catenin and β-Catenin (figure 6.1).

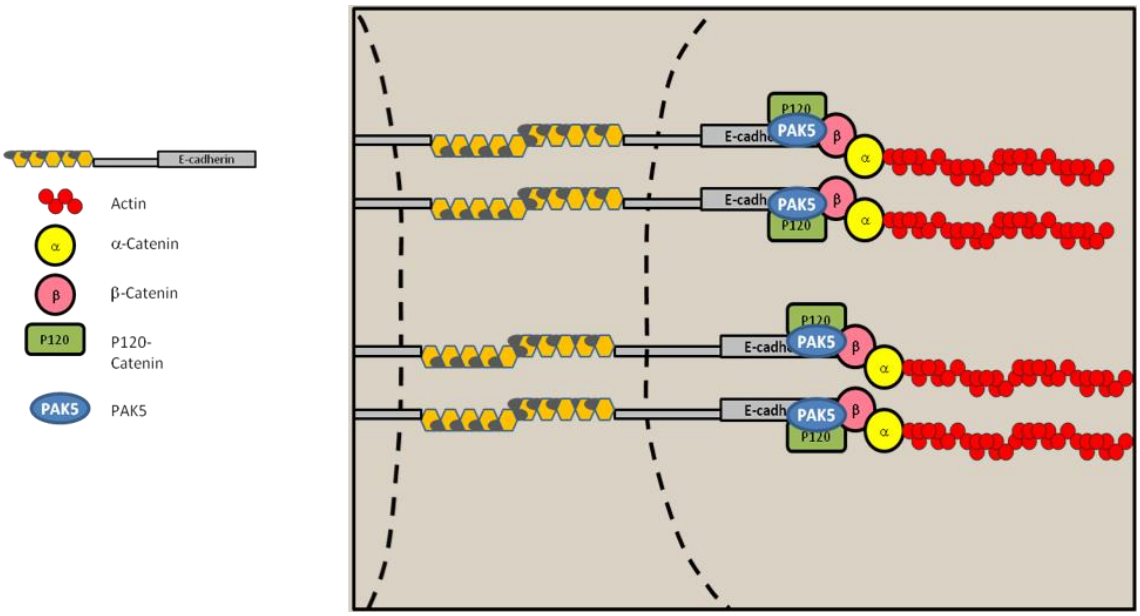


Figure 6-1 Schematic representation of PAK5 interaction with E-cadherin, p120-catenin and β-catenin at cell-cell adherens junction.

Downregulation of PAK5, as previously demonstrated (section 4.2.10), resulted in protein loss of E-Cadherin and p120-catenin. In addition, although presented in a different context, PAK5 downregulation by siRNA in HepG2 (well differentiated, polarised hepatocellular carcinoma) cells has been demonstrated to reduce the protein expression of β-catenin (Fang, Jiang et al. 2014). Binding of p120-catenin and b-Catenin to E-Cadherin cytoplasmic domain stabilises the cadherin complex by preventing cadherin internalisation and degradation (Huber, Stewart et al. 2001, Huber and Weis 2001, Davis, Ireton et al. 2003, Xiao, Allison et al. 2003). The findings of my study, using PAK5 siRNA and co-immunoprecipitation assays in RT4 and RT112 cells, and an independent study by Fang et al (Fang, Jiang et al. 2014) suggest that PAK5 contributes to support the stability of the adherens junction by maintaining a steady state and availability of adherens junction proteins at the cell membrane to maintain stable cell-cell adhesion and epithelial morphology. In contrast to previously reported reports where PAK5 expression was associated with tumour progression and cancer metastasis, I propose a role for PAK5 as a suppressor of EMT-related invasion and metastasis in bladder cancer.

Potential application of PAK5 research findings in bladder cancer

The clinical outcomes related to cancer diagnosis and treatment for many cancers have improved over the last decades but not so for bladder cancer, for which mortality has changed very little in the last few decades in England and Wales (Shah, Rachet et al. 2008). Most patients with muscle invasive bladder cancer (MIBC) will still succumb to the disease associated with metastasis despite radical treatment regimens (Gakis, Efstathiou et al. 2013, Sternberg, Bellmunt et al. 2013), while patients with non-muscle invasive bladder cancer (NMIBC) are faced with lifelong surveillance due to the characteristic high recurrence rate of urothelial cancer (Burger, Oosterlinck et al. 2013). Although the molecular events that characterise urothelial carcinoma are increasingly defined and our understanding of the relevant pathways and networks has evolved, it remains surprising that no significant markers either for diagnosis of MIBC or NMIBC, let alone a marker able to risk stratify recurrence or relative prognosis, have gained widespread acceptance and consistent validity.

One of the proteins that can potentially differentiate urothelial tumours into low or high risk of disease progression is E-Cadherin. E-Cadherin is the major mediator of cell-cell adhesion in epithelial tissues, and is expressed by most epithelial cells (Saito, Tucker et al. 2012). Decreased E-cadherin immune-reactivity was first described in bladder cancer in 1993 (Bringuier, Umbas et al. 1993). A number of studies then followed, which demonstrated cadherin switching in the setting of bladder cancer, associated with late stage, high grade disease (Rieger-Christ, Cain et al. 2001, Clairotte, Lascombe et al. 2006, Lascombe, Clairotte et al. 2006, Bryan, Atherfold et al. 2008, Mandeville, Silva Neto et al. 2008). A detailed study on cadherin switching using pT1 and T2-T3 bladder tumours (Lascombe, Clairotte et al. 2006) demonstrated that N-Cadherin expressing bladder cancer progressed more rapidly, and the majority of T2-T3 tumours demonstrated no expression of E-cadherin.

In my research to investigate the role of PAKs and bladder cancer, I have discovered the positive correlation between PAK5 and E-Cadherin protein expression in well and moderately differentiated urothelial cancer cell lines (RT4 and RT112) which form cell-cell adherens junctions. Conversely, low or undetectable protein expression of PAK5 was associated bladder cancer cell lines with mesenchymal phenotype, lacking in cell-cell adherens junctions (T24, TCCSUP and 253J). Indirect immunofluorescence studies of endogenous proteins by confocal microscopy

showed that PAK5 colocalised with E-Cadherin and p120-catenin at cell-cell adherens junctions. Upon growth factor stimulation, which resulted in E-cadherin disengagement from the cell membrane, E-Cadherin colocalised with PAK5 in punctate distribution, which suggests that these proteins may share a common pathway of transport in endocytosis.

Further investigations to characterise the function of PAK5 in bladder cancer cells with well differentiated epithelial phenotype revealed a novel role for PAK5 to support a steady state of E-Cadherin protein levels in the cells, and maintenance of stable cell-cell epithelial junction (figure 6), as siRNA knockdown of PAK5 in RT4 cells resulted in decreased protein levels of E-Cadherin and p120-Catenin. In addition, PAK5 was demonstrated to interact with p120-Catenin and β-Catenin, well-characterised proteins which binds to the cytoplasmic domain of E-Cadherin in stable adherens junction complex.

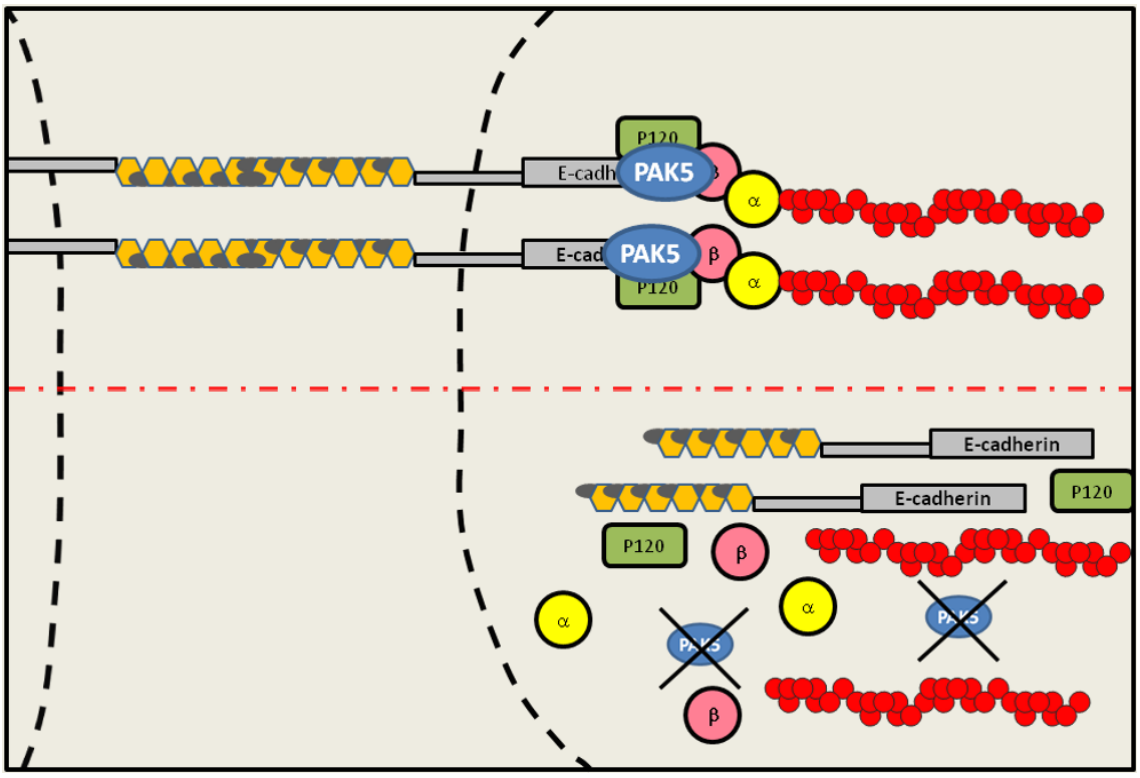


Figure 6-2: Proposed mechanism for Cadherin/Catenin engagement at the cell membrane in association with PAK5 (upper section) and uncoupling of the adherens junction complex upon PAK5 downregulation.

There are still a number of questions that remain unanswered for the mechanism on how PAK5 is involved in the regulation of E-Cadherin and cell-cell adherens junctions. Although I have elucidated that the N-terminal (regulatory domain) PAK5 binds more avidly to E-cadherin compared to C-terminal (kinase domain) PAK5, it is still unclear whether the scaffolding function of PAK5 upon engagement with E-Cadherin is associated with serine/threonine phosphorylation of E-Cadherin or the Catenins within the protein complex in the endogenous system. The uncertainty arises as the N-terminal of PAK5 contains several subcellular localisation sequences, as well as the PBD: AID domain, and its deletion as seen in C-PAK5 ablates the spatial regulation of PAK5, where it may not engage and consequently phosphorylate its downstream target.

In contrast to well characterised tyrosine phosphorylation of E-Cadherin and Catenins which results in decreased cell-cell adhesions, the evidence for PAK modulation of adherens junctions by direct serine/threonine phosphorylation of E-Cadherin or p120-/β- Catenins is limited. Although p120-Catenin has been shown to be a substrate of PAK4 (phosphorylation of T310 and S288) and PAK5 (phosphorylation of S288), the role for these interactions remain to be characterised (Wong, Reynolds et al. 2010). The study by Wong et al also highlighted the difference in subcellular localisation of PAK4 and PAK5 in their interactions with a common substrate. In addition, PAK4 interaction with p120-catenin was only observed when PAK4 was in constitutively active state (PAK4NE), whereas wild-type PAK5 and constitutively active PAK5 both have similar binding affinity and phosphorylation intensity to p120-Catenin. Although a common substrate and phosphorylation site on p120 may indicate functional redundancy of PAK4 and PAK5, it can also be speculated that these proteins may compete for the same phosphorylation site, and competitively inhibit one another to exert different cellular functions and morphology.

Further work is required to characterise the interaction between PAK5 and adherens junction proteins. In carcinomas, morphological transition known as epithelial to mesenchymal transition (EMT) has been proposed to play a significant role in tumour invasion and metastasis (Hanahan and Weinberg 2011). Key targets of the pathways that induce EMT include E-cadherin and the catenins that form the core of the adherens junctions. The mechanisms by which these proteins may be affected are diverse. E-cadherin may be downregulated by transcriptional or epigenetic silencing (Matsumura, Makino et al. 2001), as well as post-translational modifications to target the protein for lysosomal degradation (Reinhold, Reimers et al. 2010). β–catenin may be targeted by proteosomal degradation, which destabilises adherens junctions, but in certain conditions

where it is no longer coupled to E-cadherin, translocates to the nucleus and acts as a transcriptional co-factor implicated in cancer progression and metastasis (Nelson and Nusse 2004).

The findings of my research on PAK5, in parallel with emerging publications, some with contradictory conclusions, are reminiscent of research findings on p120-Catenin, which was initially identified as a substrate of the oncogenic non-receptor tyrosine kinase Src (Reynolds, Roesel et al. 1989). Ironically, although much is known about p120, its roles in cancer remain mysterious, with several lines of evidence suggesting that p120 is both a tumour suppressor and metastatic promoter (Shimazui, Schalken et al. 1996, Syrigos, Karayiannakis et al. 1998, Nakopoulou, Zervas et al. 2000, Reynolds and Roczniak-Ferguson 2004, Silva Neto, Smith et al. 2008, Soto, Yanagisawa et al. 2008, Yanagisawa, Huveldt et al. 2008, Schackmann, Tenhagen et al. 2013).

In contrast to previous reports where PAK5 expression was associated with tumour progression and cancer metastasis, I propose a role for PAK5 as a suppressor of EMT-related invasion and metastasis in bladder cancer.

Future work

The novel findings of my research on PAK5 in bladder cancer could provide the basis for further work on the role of PAK5 in the regulation of cell-cell adherens junction Cadherin-Catenin protein complex. With further resources in time and funding, I would like to take the work further by generating and inducible PAK5 knockdown system to investigate the role of PAK5 in epithelial cells in greater detail. Further research on the interaction between PAK5 and E-Cadherin/p120-Catenin using biosensor technology may also be beneficial to quantitatively examine in real-time, the role of phosphorylation on protein-protein interactions in signalling pathways. Further characterisation of PAK5 protein expression on validated bladder cancer tissue samples, in conjunction with expression and localisation of E-Cadherin or p120-Catenin will also move the research further towards the clinical translation of my work.

Appendices

Appendix 1: STR analysis for cell line authentication

BIOSYNTHESIS
DNA IDENTITY TESTING CENTER

Post Office Box 28
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Phone: 972.420.8505
Toll Free: 800.227.0627
Fax: 972.420.0442
info@biosyn.com
www.biosyn.com

Case Number: CL131171

STR Analysis for Cell Line Authentication

Institute	Researcher Name	Cell Line ID	Date Received	Date Analyzed
The Inst of Cancer Research Chester Beatty Laboratory	Radhika Patel	CL131171	7/1/2013	7/3/2013

Results:

Genetic Locus	CL131171ID1 (T24)	CL131171ID2 (5637)	CL131171ID3 (RT4)	CL131171ID4 (TCCSUP)	CL131171ID5 (RT-112)	CL131171ID6 (235J)
D8S1179	14,14	-	13,15	13,13	13,15	-
D21S11	29,29	-	30,32.2	27,31.2	27,30	-
D7S820	10,11	-	9,12	8,9	11,12	-
CSF1PO	10,12	-	10,12	10,10	10,11	-
D3S1358	16,16	-	15,15	15,16	15,15	-
TH01	6,6	-	9,9.3	6,9.3	7,7	-
D13S317	12,12	-	8,8	11,14	13,14	-
D16S539	9,9	-	9,9	9,11	11,13	-
D2S1338	20,23	-	18,19	17,17	17,24	-
D19S433	13,14	-	13,13	14,14	13,15	-
vWA	17,17	-	14,17	14,16	14,17	-
TPOX	8,11	-	8,11	8,8	8,11	-
D18S51	16,18	-	15,17	15,15	15,17	-
AMEL	X,X	-	X,Y	X,X	X,X	-
D5S818	10,12	-	11,12	12,12	10,13	-
FGA	22,22	-	22,24	21,21	25,25	-

Table 1

Conclusion:

The DNA profile obtained from item **CL131171ID1 (T24)** is compiled in Table 1.

This item yielded a full DNA profile, and the AMEL locus amplified from the X chromosome. Therefore it can be concluded that the DNA of this cell line most likely originated from a female individual. **Contamination by another human cell line was not detected.**

The DNA profile obtained from item **CL131171ID2 (5637)** is compiled in Table 1.

This item yielded **NO** DNA profile. The sample might be degraded or non-human cell line.

The DNA profile obtained from item **CL131171ID3 (RT4)** is compiled in Table 1.

This item yielded a full DNA profile, and the AMEL locus amplified from the X and Y chromosomes. Therefore it can be concluded that the DNA of this cell line most likely originated from a male individual. **Contamination by another human cell line was not detected.**

The DNA profile obtained from item **CL131171ID4 (TCCSUP)** is compiled in Table 1.

This item yielded a full DNA profile, and the AMEL locus amplified from the X chromosome. Therefore it can be concluded that the DNA of this cell line most likely originated from a female individual. **Contamination by another human cell line was not detected.**

The DNA profile obtained from item **CL131171ID5 (RT-112)** is compiled in Table 1.

This item yielded a full DNA profile, and the AMEL locus amplified from the X chromosome. Therefore it can be concluded that the DNA of this cell line most likely originated from a female individual. **Contamination by another human cell line was not detected.**

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Case Number: CL131171

STR Analysis for Cell Line Authentication

Institute	Researcher Name	Cell Line ID	Date Received	Date Analyzed
The Inst of Cancer Research Chester Beatty Laboratory	Radhika Patel	CL131171	7/5/2013	7/9/2013

Results:

Genetic Locus	CL131171ID2 (5637) redo	CL131171ID6 (235J) redo
D8S1179	-	11,11
D21S11	-	28,29
D7S820	-	9,9
CSF1PO	-	10,12
D3S1358	-	15,15
TH01	-	6,9.3
D13S317	-	9,9
D16S539	-	9,12
D2S1338	-	16,25
D19S433	-	12,15
vWA	-	17,18
TPOX	-	8,11
D18S51	-	11,13
AMEL	-	X,Y
D5S818	-	10,12
FGA	-	23,24
		Table 1

Conclusion:

The DNA profile obtained from item **CL131171ID2 (5637) redo** is compiled in Table 1.

This item yielded **NO** DNA profile. The sample might be degraded or non-human cell line.

The DNA profile obtained from item **CL131171ID6 (235J) redo** is compiled in Table 1.

This item yielded a full DNA profile, and the AMEL locus amplified from the X and Y chromosomes. Therefore it can be concluded that the DNA of this cell line most likely originated from a male individual. **Contamination by another human cell line was not detected.**

BIO-SYNTHESIS, INCORPORATED and the DNA IDENTITY TESTING LABORATORY expressly disclaim any and all responsibility for the validity of this report. The collection of samples from the named cell lines was not witnessed, nor identities verified in compliance with established chain of custody guidelines. Therefore, the results of this test should not be considered legally valid.

Deoxyribonucleic acid (DNA) was extracted and genetic loci were amplified from samples provided to the DNA Identity Testing Laboratory of Bio-synthesis, Inc. the conclusion has been verified as true and correct relative to the DNA profiles presented herein and the biographical data provided by the customer.

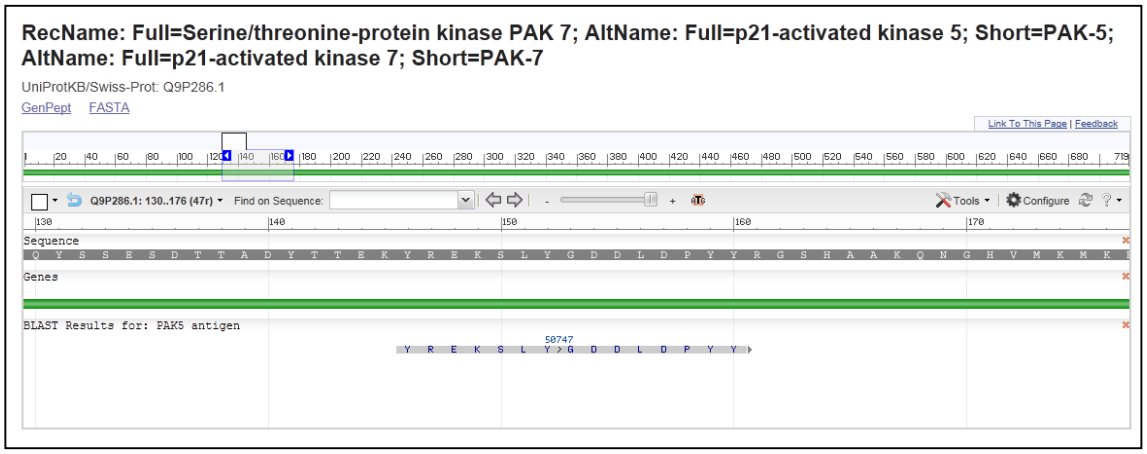
Xiaoyun Liu, Ph.D.
Associate Director
7/9/2013

Sworn and Subscribed before me this
9th day of July, 2013

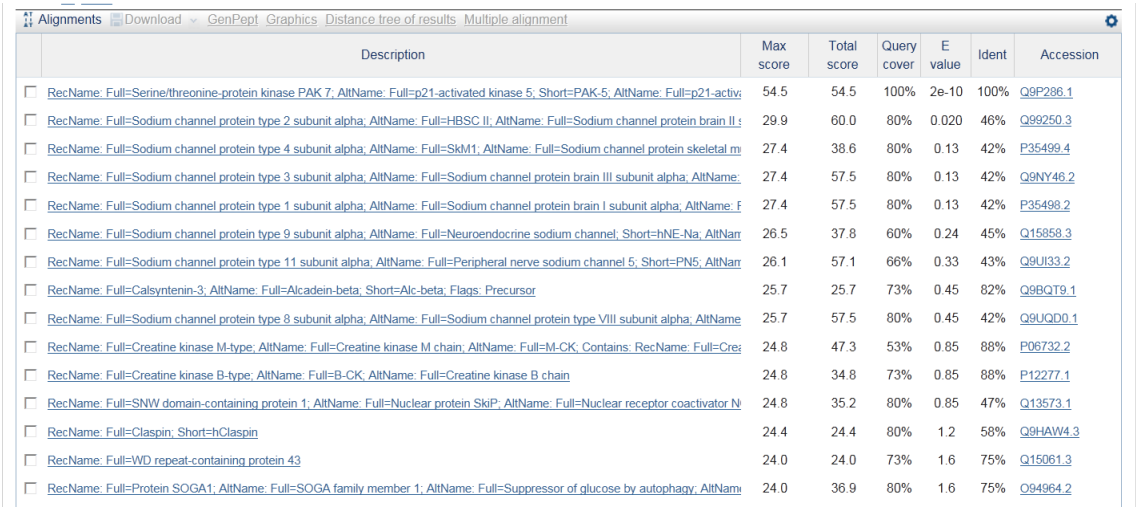
Notary Public, State of Texas

HEIDI FAZELI
Notary Public, State of Texas
My Commission Expires
October 21, 2014

Appendix 2

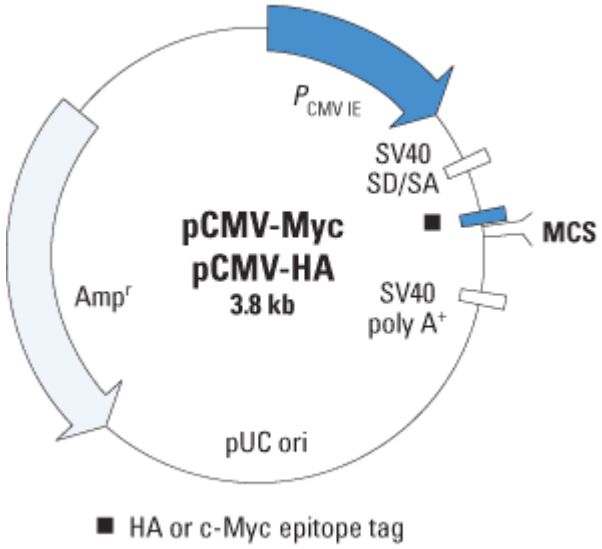


Appendix 3

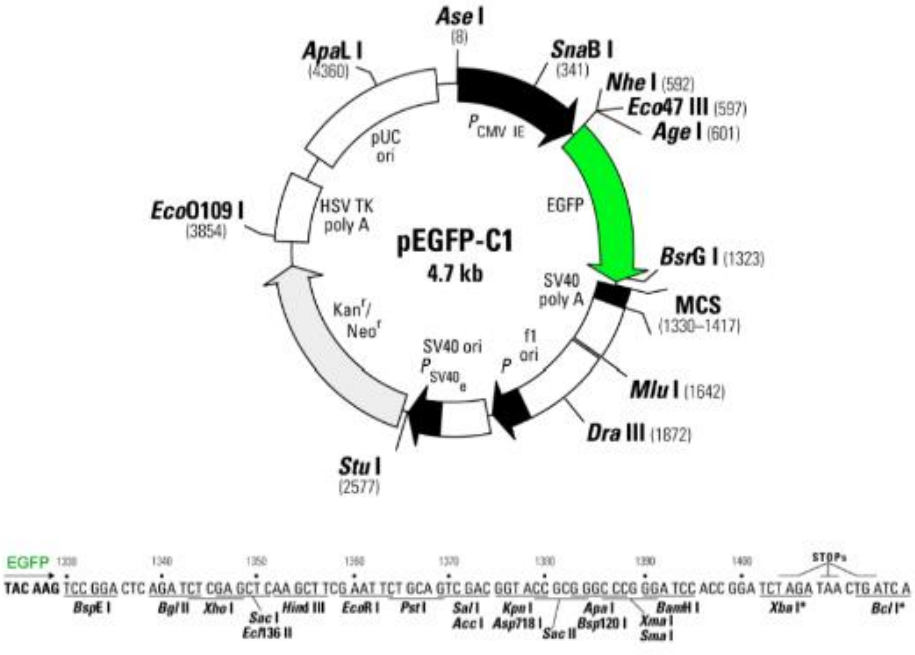


Description	Max score	Total score	Query cover	E value	Ident	Accession
RecName: Full=Serine/threonine-protein kinase PAK 7; AltName: Full=p21-activated kinase 5; Short=PAK-5; AltName: Full=p21-activ	54.5	54.5	100%	2e-10	100%	Q9P286.1
RecName: Full=Sodium channel protein type 2 subunit alpha; AltName: Full=HBSC II; AltName: Full=Sodium channel protein brain II	29.9	60.0	80%	0.020	46%	Q99250.3
RecName: Full=Sodium channel protein type 4 subunit alpha; AltName: Full=SkM1; AltName: Full=Sodium channel protein skeletal m	27.4	38.6	80%	0.13	42%	P35499.4
RecName: Full=Sodium channel protein type 3 subunit alpha; AltName: Full=Sodium channel protein brain III subunit alpha; AltName:	27.4	57.5	80%	0.13	42%	Q9NY46.2
RecName: Full=Sodium channel protein type 1 subunit alpha; AltName: Full=Sodium channel protein brain I subunit alpha; AltName: F	27.4	57.5	80%	0.13	42%	P35498.2
RecName: Full=Sodium channel protein type 9 subunit alpha; AltName: Full=Neuroendocrine sodium channel; Short=hNE-Na; AltNam	26.5	37.8	60%	0.24	45%	Q15858.3
RecName: Full=Sodium channel protein type 11 subunit alpha; AltName: Full=Peripheral nerve sodium channel 5; Short=PN5; AltNam	26.1	57.1	66%	0.33	43%	Q9UI33.2
RecName: Full=Calsyntenin-3; AltName: Full=Alcadein-beta; Short=Alc-beta; Flags: Precursor	25.7	25.7	73%	0.45	82%	Q9BQT9.1
RecName: Full=Sodium channel protein type 8 subunit alpha; AltName: Full=Sodium channel protein type VIII subunit alpha; AltName	25.7	57.5	80%	0.45	42%	Q9UQD0.1
RecName: Full=Creatine kinase M-type; AltName: Full=Creatine kinase M chain; AltName: Full=M-CK; Contains: RecName: Full=Crea	24.8	47.3	53%	0.85	88%	P06732.2
RecName: Full=Creatine kinase B-type; AltName: Full=B-CK; AltName: Full=Creatine kinase B chain	24.8	34.8	73%	0.85	88%	P12277.1
RecName: Full=SNW domain-containing protein 1; AltName: Full=Nuclear protein SkiP; AltName: Full=Nuclear receptor coactivator N	24.8	35.2	80%	0.85	47%	Q13573.1
RecName: Full=Claspin; Short=hClaspin	24.4	24.4	80%	1.2	58%	Q9HAW4.3
RecName: Full=WD repeat-containing protein 43	24.0	24.0	73%	1.6	75%	Q15061.3
RecName: Full=Protein SOGA1; AltName: Full=SOGA family member 1; AltName: Full=Suppressor of glucose by autophagy; AltNam	24.0	36.9	80%	1.6	75%	O94964.2

Appendix 4: Vector maps



Clontech pRK5 Myc vector (modified for Gateway™ Cloning system)



Clontech pEGFP-C3 vector (modified for Gateway™ Cloning system)

SiRNA oligonucleotide sequences

Target	Supplier	Sequence	Location	
Control/ non silencing	Qiagen	AAT TCT CCG AAC GTG TCA CGT		
Pak5	Dharmacon		3' UTR	Si1
	Dharmacon		3' UTR	Si2
	Dharmacon		3' UTR	Si3
	Dharmacon		3' UTR	Si4
	Qiagen	ATG ATC TGG ATC CGT ATT ATA	Coding region	Si63
	Qiagen	ATG GTG TGC ACG TTT CAT TAA	3' UTR	Si70

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